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EDITED BY

SIMON FLEXNER, M.D. PEYTON ROUS, M.D.

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SEROLOGICAL DIFFERENTIATION OF STERIC ISOMERS.

By K. LANDSTEINER, M.D., and J. van der SCHEER.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, May 29, 1928.)

In previous work[1] the conclusion was arrived at that serological specificity depends on the chemical constitution of the reacting groups in general and probably also on their spatial configuration. The latter assumption had been made on account of the reactions observed with antigens containing aromatic compounds substituted in various positions of the benzene nucleus. The experiments dealt with in the present paper were carried out in order to secure conclusive evidence for this conception. Thus we endeavored to determine whether optical isomers can be differentiated by means of serum reactions.

The substances chosen for this purpose were *l*- and *d*-phenyl (para-aminobenzoylamino) acetic acid. These compounds had been used by Ingersoll and Adams[2] in an investigation on the affinity to fibers, of optically isomeric dyes. By diazotization and coupling to dimethylaniline they obtained two isomeric dyes which were absorbed by wool in different amounts; when β-naphtol was used in place of dimethylaniline the dyes were absorbed equally well.

The preparation of the phenyl (para-aminobenzoylamino) acetic acid was carried out in the main according to the directions of Ingersoll and Adams. Phenyl-aminocyanide made by the interaction of benzaldehyde, sodium cyanide, and ammonium chloride in water solution, was hydrolyzed to phenylaminoacetic acid.[3] The *d*- *l*- phenylaminoacetic acid was resolved into the levo and dextro product by means of fractional crystallization of the *d*-camphor sulfonate and *l*-camphor sulfonate respectively. In the recrystallization of the *l*-amino acid *d*-camphor

[1] A review of the subject can be found in The chemical aspects of immunity, by H. Gideon Wells, American Chemical Society, Monograph Series, New York, 1925, 77.

[2] Ingersoll, A. W., and Adams, R., *J. Am. Chem. Soc.*, 1922, xliv, 2930; 1925, xlvii, 1169.

[3] *Cf.* Marvel, C. S., and Noyes, W. A., *J. Am. Chem. Soc.*, 1920, xlii, 2264.

sulfonate it was found of advantage to filter off the crystals when the solution had cooled to about 45°. Few recrystallizations were sufficient to obtain a pure product. For the isolation of the *d*-amino acid, inactive camphor sulfonic acid was used. The *d*-amino acid *l*-camphor sulfonate was purified by several recrystallizations.

Both forms of phenylaminoacetic acid were condensed with para-nitrobenzoyl-chloride to form *l*- and *d*-phenyl (*p*-nitrobenzoylamino) acetic acid $HO_2CC(C_6H_5)$-$HNHCOC_6H_4NO_2$. For recrystallization of this substance thirty times its weight of a boiling mixture of one part of alcohol and two parts of water was used. Crystallization was allowed to take place while stirring. The melting points agreed with those given by the authors named. The nitro compounds were reduced to *l*- and *d*-phenyl (para-aminobenzoylamino) acetic acid by means of ferrous sulfate and ammonia and the final products were recrystallized from water. The levo form had the melting point 168–169°, 0.400 gm. dissolved and made up to 5 cc. with normal HCl gave at 20° a rotation of −14.97 in a 2 dm. tube, with sodium light. For this concentration Ingersoll and Adams give a rotation of −15.00 and the melting point 168–169°. The dextro form had a melting point of 168–169°; 0.400 gm. dissolved and brought up to a volume of 5 cc. with normal HCl at 20° gave a rotation of +14.98 in a 2 dm. tube with sodium light, in accordance with the data of Ingersoll and Adams.

d-l-Phenyl (para-aminobenzoylamino) acetic acid was prepared in the manner described by the authors quoted, from the inactive phenylaminoacetic acid. Melting point 152°.

The levo- and dextro-phenyl (para-aminobenzoylamino) acetic acids will for the sake of convenience be designated as *l*- and *d*-acid. The azoproteins prepared from these amino acids by diazotization and coupling to proteins will be referred to as *l*- and *d*-antigens and likewise the sera obtained by immunization with the azoproteins as *l*- and *d*-immune sera, respectively; *i* denotes the inactive preparations.

Preparation of the l- and d-Antigens for Immunization.—The *l*- and *d*-acids were coupled to proteins in the following manner: 17.12 gm. of the optically active compound were dissolved in 200 cc. water and 25 cc. of 7 normal HCl and diazotized with the required amount of sodium nitrite at a temperature of 0–5°C. with starch iodide paper as indicator; the diazo solution was diluted with ice water to a volume of 500 cc. and poured into a mixture of 500 cc. of horse serum and 500 normal sodium carbonate; coupling was allowed to take place for 10 minutes at 0–5°. By acidification with hydrochloric acid the azoprotein was precipitated and after filtration it was dissolved in a small volume of water by addition of a little normal sodium carbonate. It was reprecipitated from this solution with a large quantity of alcohol and enough hydrochloric acid to flocculate the material. The precipitate was filtered, again treated in the same manner, and the azoprotein was freed from alcohol by redissolving in water and sodium carbonate, and precipitation with hydrochloric acid. It was finally dissolved and brought to a volume of 950 cc. using just enough sodium carbonate to give neutral reaction to litmus

TABLE I.

To 0.2 cc. of the diluted antigen were added 3 capillary drops of *l*-immune serum.

Readings taken after:	*l*-Antigen prepared with chicken serum Dilution 1:				*d*-Antigen prepared with chicken serum Dilution 1:			
	20	100	500	2500	20	100	500	2500
2 hrs. at room temperature	++	+±	+	tr.	+±	0	0	0
Night in ice box	+++	++	++	+±	++	±	f.tr.	0

TABLE II.

To 0.2 cc. of the diluted antigen were added 4 capillary drops of *d*-immune serum.

Readings taken after:	*l*-Antigen made with chicken serum Dilution 1:				*d*-Antigen made with chicken serum Dilution 1:			
	20	100	500	2500	20	100	500	2500
2 hrs. at room temperature	++	0	0	0	+±	+±	+±	±
Night in ice box	+++	±	0	0	+++	+++	++	+

TABLE III, *a*.

To 0.2 cc. of the diluted antigens were added 3 capillary drops of *l*-immune serum.

Readings taken after:	*l*-Antigen made with chicken serum Dilution 1:		*d*-Antigen made with chicken serum Dilution 1:		*i*-Antigen made with chicken serum Dilution 1:	
	100	500	100	500	100	500
2 hrs. at room temperature	+	+	0	0	+	±
Night in ice box	++±	++	±	0	++±	+±

TABLE III, *b*.

To 0.2 cc. of the diluted antigens were added 2 capillary drops of *d*-immune serum.

Readings taken after:	*l*-Antigen made with chicken serum Dilution 1:		*d*-Antigen made with chicken serum Dilution 1:		*i*-Antigen made with chicken serum Dilution 1:	
	100	500	100	500	100	500
2 hrs. at room temperature	f.tr.	0	+	+	+	±
Night in ice box	+	0	++	++	++	++

and the necessary amount of a salt solution to make the ultimate salt concentration approximately 1 per cent. 50 cc. of a 5 per cent phenol solution was added.

TABLE IV, *a*.

0.2 cc. of *l*-antigen (diluted 1:500) prepared with chicken serum was mixed with 0.05 cc. of a neutral solution containing 1 millimol of the substances indicated, in 10 cc. To this 3 capillary drops of *l*-immune serum were added. The control tube contains only immune serum and antigen.

Readings taken after:	1	2	3	4	5	6	7	8	*l*-Acid	*d*-Acid	*i*-Acid	Control
3 hrs. at room temperature	±	+	+	+	±	+	±	+	0	±	f.tr.	+±
Night in ice box	+±	+±	++	++	++	+±	+±	++	tr.	+±	+	++

TABLE IV, *b*.

0.2 cc. of *d*-antigen (diluted 1:500) prepared with chicken serum was mixed with 0.05 cc. of a neutral solution containing 1 millimol of the substances indicated in the first line of the table in 10 cc. To this mixture 3 capillary drops of *d*-immune serum were added.

Readings taken after:	1	2	3	4	5	6	7	8	*l*-Acid	*d*-Acid	*i*-Acid	Control
3 hrs. at room temperature	+	+	+±	+±	+	+	+	+±	±	0	0	+±
Night in ice box	++	++	++	++	++	++	+±	++±	+±	0	+	++±

TABLE IV, *c*.

The following tests were made like those given in Tables IV,*a* and IV,*b*, but 0.05 cc. of solution containing 0.5 millimol of the *d*-, *l*-, and *i*-acids was added.

Readings taken after:	*l*-Antigen and 3 capillary drops *l*-immune serum, with addition of:				*d*-Antigen and 3 capillary drops *d*-immune serum, with addition of:			
	l-Acid	*d*-Acid	*i*-Acid	Control	*l*-Acid	*d*-Acid	*i*-Acid	Control
3 hrs. at room temperature	0	±	0	+	+	0	±	+±
Night in ice box	±	++	+±	++	++	tr.	+	++±

Immunization.—Two series of seven rabbits each were injected with 15 cc. of the *l*- and *d*-antigens at weekly intervals. In each of the two series one serum of medium strength was produced and in addition some weakly reacting sera.

Antigens for the Tests.—These were prepared in the same way as the antigens for immunization; as protein component chicken serum was used. After coupling, the azoprotein was precipitated with acid, washed with water, and brought into solution by means of sodium carbonate. The quantity of antigen in the solution was determined by precipitation with alcohol and weighing of the dried substance. The dilutions given in the tables are in terms of a 5 per cent stock solution.

The intensity of the reactions is indicated as follows: 0, f.tr. (faint trace), tr. (trace), ±, +, + ±, etc.

The immune sera obtained were tested against the *l*-, *d*-, and *i*-antigens with the results given in Tables I, II, and III *a*, *b*.

In addition to the tests presented others were made in order to determine the inhibiting effect on the precipitin reaction,[4] by the *l*-, *d*-, and *i*-acids and other substances, namely; (1) benzoic acid; (2) para-aminobenzoic acid; (3) sulfanilic acid; (4) acetic acid; (5) phenylacetic acid; (6) hippuric acid; (7) phenylglycocoll; (8) phenylalanine (Tables IV,*a*, IV,*b*, IV,*c*).

DISCUSSION AND SUMMARY.

The tests presented in Tables I, II, and III show that only in the higher concentration cross reactions do take place, and that there is definite specificity of the two sorts of immune sera for the homologous antigens. Thus it is easy to differentiate the *l*- and *d*-antigens in dilutions 1:100 and upwards. The occurrence of cross reactions can readily be ascribed to the fact that the *l*- and *d*-acids present in the two antigens are identical in every respect but the position of the groups connected with the asymetric carbon atom. The *i*-antigen reacts with both sorts of immune sera as could be expected since it must consist of a mixture of equal parts of *l*- and *d*-antigen.

The reactions of the *i*-antigen appear to be only slightly weaker than those of the homologous ones owing to the fact that the intensity of the reactions diminishes but slowly with increasing dilution of the antigens. It is also to be considered that small differences cannot be judged very accurately.

Tests with two *l*- and four *d*-immune sera[5] not recorded in the tables confirmed the results already discussed.

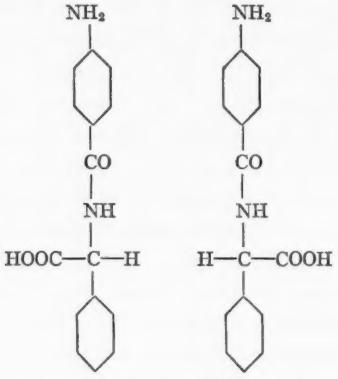
[4] *Cf.* Landsteiner, K., *Biochem. Z.*, 1920, civ, 280.

[5] Two of these, of medium strength, were obtained from an additional immunization experiment.

Considering that ferments are known to be adapted ordinarily to one type of steric isomers it may be worth noting that antibodies were formed by the same species of animals for optical antipodes.

From the results summarized in Tables IV,*a* and IV,*b* one sees that the *l*- and *d*-immune sera also differentiate clearly between the *l*- and *d*-acids when they are not diazotized and not combined with protein. The *l*-acid inhibits much more the precipitation of the *l*-antigen by the homologous immune serum than the *d*-acid and the converse effect occurs if the inhibiting action is tested on the precipitation of *d*-antigen by *d*-immune serum. The inactive phenyl (para-aminobenzoylamino) acetic acid behaved in such tests as a mixture of *l*- and *d*-acids, *i.e.*, it acted markedly in both cases, more than the heterologous and less than the homologous acid.

The experiments reported bring a definite proof for the view that the steric configuration of antigenic groups is one of the factors determining serological specificity. In the particular case under consideration the mere difference in the position of H and COOH as indicated in the following formulas sufficed to alter the reactivity.



The fact that steric isomers are acted upon selectively by immune sera may be supposed to play a significant part in the serological specificity of carbohydrates such as those discovered in bacterial antigens.

THE INFLUENCE OF CHOLESTEROL ON EXPERIMENTAL TUBERCULOSIS.

By RICHARD E. SHOPE, M.D.

(From the Department of Pharmacology of the State University of Iowa Medical School, Iowa City, Iowa, and the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

(Received for publication, June 1, 1928.)

It is generally recognized today that the high tuberculosis morbidity and mortality rates among the white races are conjoined with phenomena which indicate that the majority of individuals possess an important, though variable, degree of resistance to the progress of this infection. The nature of this resistance is very obscure. Analogy with more acute diseases suggests the possibility that the mechanisms of acquired immunity as represented by antibody formation and action, phagocytosis, or the still undefined "tissue resistance" may be factors of importance. Such attempts as have been made to determine the place of these factors have led to no decisive results.

The consecutive and rather characteristic series of tissue reactions leading to the formation of the tubercle, the degeneration of the tubercle, and, in favorable cases, to the walling off, fibrosis, or calcification of these abnormal structures and their associated bacilli, suggest as another major possibility that substances chemically less elusive than antibodies, and with a more direct bactericidal action against the tubercle bacillus might be formed or accumulated in the diseased tissue and there act as adjuvants in the healing process. If this were the case, it might be hoped that the recognition of such substances would make possible their artificial quantitative or qualitative alteration with beneficial results.

In reviewing the reported chemical analyses of tuberculous tissues, the most striking variation from the normal is the high value frequently obtained for cholesterol.

Caldwell (1) has conducted the most complete analysis of tuberculous tissues using modern quantitative methods. He found that in normal bovine lymph

glands cholesterol made up 6.52 per cent of the lipin fraction, or 1.58 per cent of the dry weight of the specimen; while in the walls of tuberculous bovine lymph glands cholesterol made up 13.17 per cent of the fatty substances, or 3.88 per cent of the dry weight. In the caseous material from mesenteric and peribronchial bovine lymph glands cholesterol made up 26.58 per cent of the total lipins, or 5.18 per cent of the dry weight of the specimen. In normal bovine liver he found the cholesterol content of the lipin fraction to be 5.07 per cent, or cholesterol made up 1.6 per cent of the dry weight of the specimen. In the lipins from the walls of liver tubercles cholesterol constituted 14.4 per cent, or 2.25 per cent of the dry specimen. The caseous material from liver tubercles was very rich in cholesterol; the lipin fraction was 26.48 per cent cholesterol, and calculated on the dry weight of the specimen cholesterol made up 4.7 per cent. From these analyses it is evident that cholesterol increases at tuberculous foci from 200 to 300 per cent, depending on the degree of caseation.

In accord with Caldwell's results are those of Jaffé and Levinson (2). These authors found that it was possible to increase further the localized cholesterol by the administration of the substance during the course of the infection. In rabbits which were fed cholesterol in cottonseed oil before and during an infection with bovine tubercle bacilli, the tuberculous lesions showed much more optically active fat than did tuberculous lesions of rabbits that received no cholesterol, indicating that, with an increase in the supply of cholesterol to the animal body, it was possible to increase further the amount of cholesterol or its esters localizing in tuberculous tissues. This they considered due to the fact that the epithelioid cells of the tubercles have their origin from the reticulo-endothelial system which has (as some suppose) to do with cholesterol metabolism. From their experiments, which, so far at least as they concerned the point, were very limited in number, they concluded that cholesterol does not protect rabbits against tuberculosis.

The experiments referred to were of such suggestive importance that they have been repeated in part, in this laboratory, with guinea pigs. The results are in essential agreement with those of previous workers.

The accumulation of cholesterol or cholesterol esters in tuberculous tissues, and especially in the actual anatomical tubercles, suggested the possibility of using this substance in some chemotherapeutic experiments. Since it appeared to be capable of penetrating, and localizing in tubercles, it seemed possible that, if compounds could be made that would contain cholesterol combined with other radicles known to possess bactericidal properties, they might prove to be of therapeutic value. To test the success of such an assumption two series of chemo-

therapeutic experiments have been conducted. In the first series of experiments cholesterol alone has been used in treating guinea pigs experimentally infected with *B. tuberculosis*. In the second series various compounds containing cholesterol or an oxidation product of cholesterol have been used.

EXPERIMENTAL.

I. Chemical.

The cholesterol used was prepared either from human gall stones or hog brains. Cholesteryl chloride was prepared using Rayman's method (3) and cholesteryl anilide and cholesteryl toluide by Walitzky's method (4). Cholesterol sulfuric acid was made according to the method of Mandel and Neuberg (5). A carboxy acid of cholesterol was prepared according to the method of Diels and Abderhalden (6), the last step in their procedure being omitted (refluxing with methyl ethyl ketone). The acid thus obtained had a melting point of 90°C. instead of 290°C. as did the one described by them.

Quinine cholesterylate was prepared by pouring quinine hydrochloride, dissolved in water, into an aqueous solution of sodium cholesterylate until no more precipitate was formed. This precipitate was washed in water and then dried.

The compounds mentioned, with the exception of the two that were water soluble (sodium cholesterylate and sodium cholesterol sulfate), were prepared for injection by dissolving in hot alcohol and pouring the hot alcoholic solution into hot distilled water with constant stirring. A rather stable suspension was obtained. This was heated, to drive off most of the alcohol and to get the suspension down to the strength desired for injection, and then filtered. The sodium salt of the carboxy acid of cholesterol, being water soluble, was used in 2 per cent solution in distilled water except where otherwise specified. Sodium cholesterol sulfate was used in 0.5 per cent solution in distilled water.

II. Biological.

A. Therapeutic Experiments with Cholesterol Alone.

Experiment 1.—(Table I, Chart 1.) 20 guinea pigs were inoculated subcutaneously in the inguinal region with 1 cc. of a very thin suspension of recently isolated human type tubercle bacilli. They were divided into 2 groups of 10 animals each. The infection was allowed to run 3 weeks and then the animals in 1 group were given 4 intraperitoneal injections, each of 4 mg. of cholesterol, suspended in distilled water, at intervals of 3 days. The animals in the control group each received 2 cc. of distilled water intraperitoneally on corresponding days. Animals that obviously died as the result of the injections were discarded. This accounted for 1 from each group. The results are given in Table I.

Experiment 2.—(Table II, Chart 2.) The guinea pigs in this experiment were inoculated subcutaneously with ¼ cc. of a very thin, paper filtered, suspension of recently isolated human type tubercle bacilli. 15 days were allowed to elapse before treatment and then the animals were divided into 3 groups. A group of 7 controls received 7 injections of 2 cc. of distilled water, intraperitoneally, at intervals of 4 days. Another 7 animals received 7 injections, each of 8.8 mg. of choles-

TABLE I.

Days of Survival Following Infection.

Control	Cholesterol	Control	Cholesterol
33	49	57	66
35	50	58	70
42	53	60	72
50	58	62	112
53	65		
Average.....		50	66

TABLE II.

Days of Survival Following Infection.

Control	Cholesterol	Irradiated cholesterol
63	80	60
64	108	66
71	110	86
75	113	98
95	117	100
96	152	103
96		123
		126
		132
		136
Average: 80	113	103

terol in water suspension, and a group of 11 animals received 7 injections, each of 10 mg. of cholesterol, that had been exposed to the rays from a mercury quartz lamp, after the method of Hess, Weinstock, and Helman (7), on corresponding days. Animals dying as a result of the injections were discarded thus eliminating 1 animal from each of the cholesterol groups. The results are tabulated in Table II.

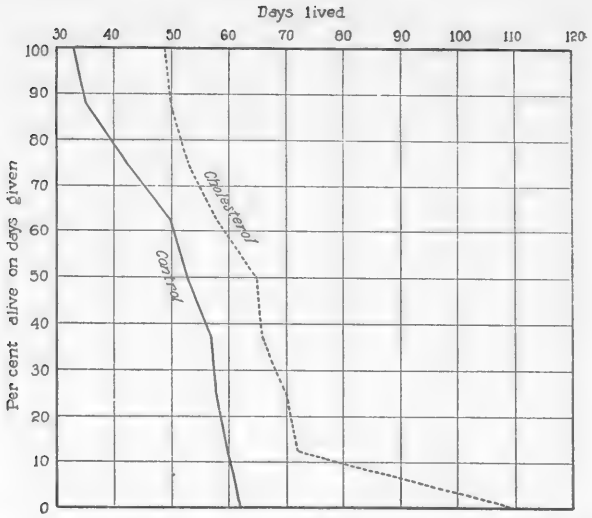


CHART 1. Therapeutic Experiment 1. 1st day. Animals given 1 cc. of a light suspension of freshly isolated tubercle bacilli (human type) subcutaneously. 18th day. Treatment started. 30th day. Treatment stopped. All groups charted.

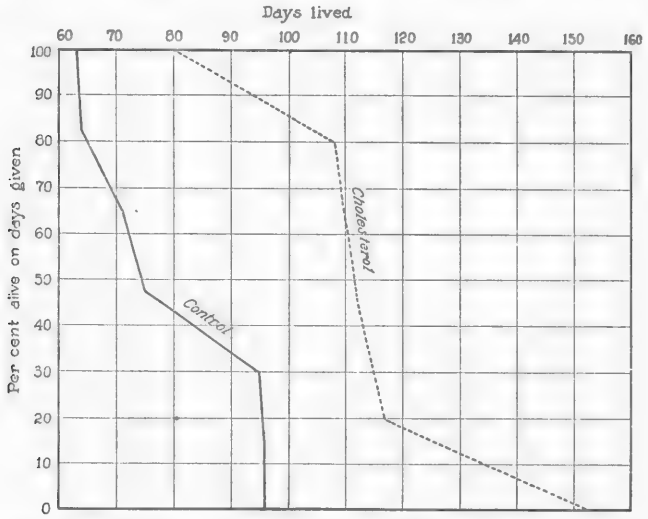


CHART 2. Therapeutic Experiment 2. 1st day. Animals given 0.25 cc. of a paper filtered suspension of tubercle bacilli (human type) subcutaneously. 14th day. Treatment started. 24th day. Treatment stopped. Groups charted: Controls and those receiving cholesterol.

The work so far reported in this paper was carried out in the Department of Pharmacology at the State University of Iowa Medical School.

The first two experiments had rather consistently indicated a favorable influence of cholesterol on a tuberculous infection in guinea pigs; but the number of animals used was too small to warrant definite conclusions. Only one type of infection had been studied, an acute tuberculosis produced by the tubercle bacillus of human type. It seemed desirable to repeat the experiments with a larger series of animals and a more chronic type of infection.

TABLE III.

Days of Survival Following Infection.

Control	Cholesterol	Control	Cholesterol
88	130	176	164
94	133	180	166
107	136	182	167
114	138	183	169
121	141	184	169
124	141	184	170
129	146	185	171
143	146	185	174
146	148	192	182
146	149	194	185
153	155	202	190
167	156	205	212
171	160	208	
172	160	239	
175	162		
Average.....		163	160

Experiment 3.—(Table III, Chart 3.) 60 guinea pigs from Family 13 (inbreds) were injected subcutaneously with 1/10 mg. of Culture H 7156, human type tubercle bacilli. The culture in this amount was known to produce a chronic type of tuberculosis in guinea pigs. The infection was allowed to progress untreated for 2 weeks. Then the animals were divided into 2 groups of 30 each. 1 group of animals received 10 intraperitoneal injections, each of 18 mg. of cholesterol in distilled water suspension, while the animals in the control group received 10 intraperitoneal injections of 3 cc. of distilled water on corresponding days. The animals were injected on the 14th, 18th, 22nd, 26th, 30th, 34th, 38th, 57th, 68th,

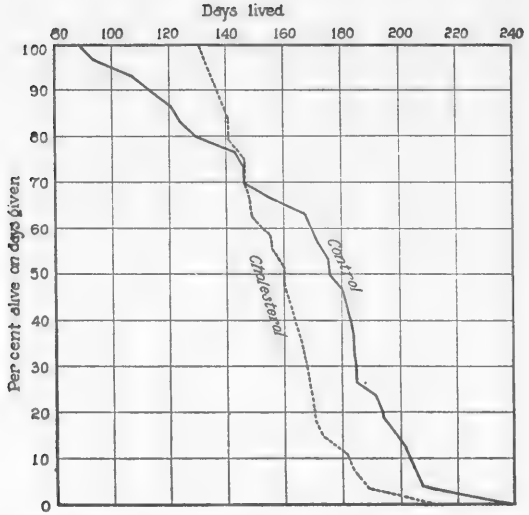


CHART 3. Therapeutic Experiment 3. 1st day. Animals given 1/10 mg. of H 7156 subcutaneously. 14th day. Treatment started. 110th day. Treatment stopped. All groups charted.

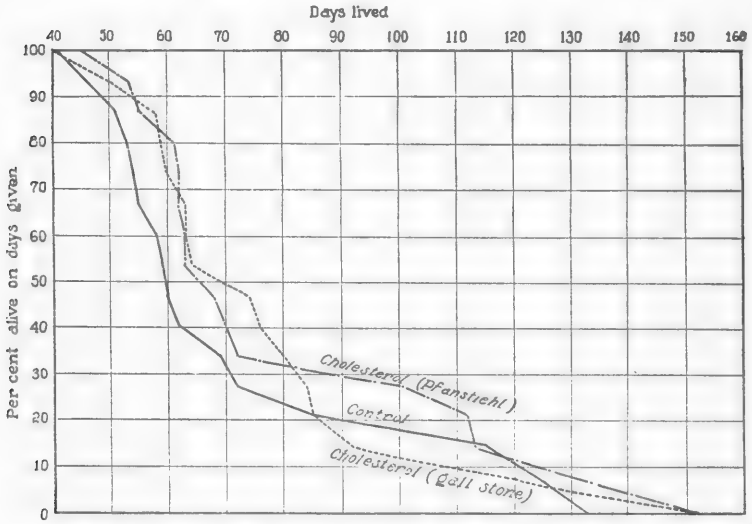

Days lived
40 50 60 70 80 90 100 110 120 130 140 150 160
100 90 80 70 60 50 40 30 20 10 0
Per cent alive on days given
Cholesterol (Pfanstiehl)
Control
Cholesterol (Gall stone)

CHART 4. Therapeutic Experiment 4. 1st day. Animals given 1 mg. of H 7156 subcutaneously. 5th day. Treatment started. 125th day. Treatment stopped. All groups charted.

and 110th days following inoculation. Animals that obviously died as a result of the injections were ruled out of the experiment. This accounted for 1 of the

TABLE IV.

Days of Survival Following Infection.

Control	Pfanstiehl cholesterol	Gall stone cholesterol
41	45	40
46	53	50
51	55	58
53	61	59
54	62	60
55	62	63
58	63	63
59	63	64
60	68	74
62	70	76
69	72	80
72	100	84
85	112	85
115	113	92
125	152*	121
133	152*	152*
Average: 71	81	76

* Killed on 152nd day to terminate experiment.

TABLE V.

Days of Survival Following Infection.

Control	Cholesterol	Control	Cholesterol
35	28	55	53
42	38	56	55
45	40	58	56
48	42	61	66
49	45	61	68
50	50	64	75
51	50	74	104
53	50	79	
Average.....		55	54.6

control group, and 3 of the 30 receiving cholesterol. The results are given in Table III.

Experiment 4.—(Table IV, Chart 4.) Another group of 48 stock guinea pigs, crossbred in heredity, were inoculated subcutaneously in the inguinal region with 1 mg. of Culture H 7156, ten times as large a dose as that previously used, with the intention of getting a relatively more acute infection that would be more comparable to the first two experiments reported in this paper. This time the infection was allowed to run only 5 days before treatment was instituted. Then the animals were divided into 3 groups of 16 each. Group 1 was kept as a control group and received 10 intraperitoneal injections of 3 cc. of sterile distilled water on the 5th, 8th, 12th, 19th, 23rd, 27th, 31st, 103rd, 111th, and 121st days following infection. Group 2 animals received 10 intraperitoneal injections, each of 21 mg.

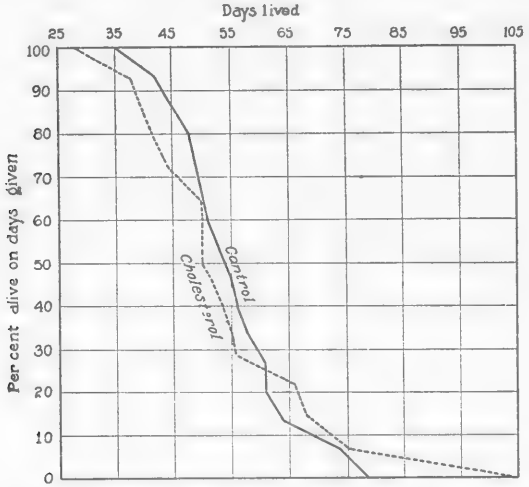


CHART 5. Therapeutic Experiment 5. 1st day. Animals given 1/5 mg. of Bovine 14 subcutaneously. 3rd day. Treatment started. 16th day. Treatment stopped. All groups charted.

of Pfanstiehl cholesterol (hog brain) in watery suspension, on corresponding days; and Group 3 animals received intraperitoneal injections of 21 mg. of cholesterol, prepared from human gall stones, on corresponding days. The results are given in Table IV.

Experiment 5.—(Table V, Chart 5.) 32 guinea pigs were inoculated subcutaneously with 1/5 mg. of a culture of Bovine 14. The infection was allowed to progress 3 days and then the animals were divided into 2 groups of 16 each and treatment was begun. The control group animals each received 5 intraperitoneal injections of 3 cc. of distilled water on the 3rd, 6th, 10th, 14th, and 19th days following infection. The second group each received 5 intraperitoneal injections,

each of 21 mg. of cholesterol, on corresponding days. 1 of the animals receiving cholesterol died of a peritonitis and was discarded. The results are given in Table V.

B. Therapeutic Experiments with Compounds Containing Cholesterol or Derivatives of Cholesterol.

Experiment 6.—(Table VI.) The guinea pigs in this experiment received 1 cc. of a very thin suspension of recently isolated human type tubercle bacilli subcutaneously. They were then divided into groups of 10 each and caged by groups. 3 weeks following inoculation treatment was begun. 1 group received 4 intraperitoneal injections, each of 3 mg. cholesteryl chloride at intervals of 3 days. The second group received 2 mg. cholesteryl anilide, the third group 1.2 mg.

TABLE VI.

Days of Survival Following Infection.

Control	Cholesteryl chloride	Cholesteryl toluide	Cholesteryl anilide
33	32	32	33
35	49	37	41
42	50	53	46
50	62	55	53
53	64	56	54
57	65	66	58
58	66	87	58
60	80	95	60
62	98		60
			85
Average: 50	63	60	55

cholesteryl toluide, and the controls 2 cc. of distilled water on corresponding days. Individuals dying during the period of the injections were discarded. The results are given in Table VI.

Experiment 7.—(Table VII.) The guinea pigs in this experiment received ¼ cc. of a very thin, paper filtered suspension of recently isolated human type bacilli subcutaneously. They were then divided into 2 groups of 7 each and 15 days were allowed to elapse before treatment was begun. Then the animals in 1 group were given 7 intraperitoneal injections, each of 10 mg. of sodium cholesterol sulfate, at intervals of 4 days. The animals in the control group received 2 cc. intraperitoneal injections of distilled water on corresponding days. The results are given in Table VII.

Experiment 8.—(Table VIII.) 60 guinea pigs from Family 13 (inbreds) were injected subcutaneously with 1/10 mg. of Culture H 7156, human type of tubercle

bacilli, and divided into 2 groups of 30 animals each. The infection was allowed to progress untreated for 2 weeks. Then the animals in 1 group were given 10 intraperitoneal injections, each of 9 mg. of quinine cholesterylate in distilled water suspension, on the 14th, 18th, 22nd, 26th, 30th, 34th, 38th, 57th, 68th, and 110th

TABLE VII.

Days of Survival Following Infection.

Control	Sodium cholesterol sulfate	Control	Sodium cholesterol sulfate
63	61	95	99
64	71	96	111
71	77	96	126
75	97		
Average.....		80	92

TABLE VIII.

Days of Survival Following Infection.

Control	Quinine cholesterylate	Control	Quinine cholesterylate
88	105	176	170
94	122	180	171
107	132	182	176
114	133	183	177
121	151	184	184
124	152	184	185
129	158	185	186
143	159	185	190
146	160	192	254
146	161	194	256*
153	162	202	
167	165	205	
171	166	208	
172	168	239	
175	169		
Average.....		163	168

* Killed on 256th day to terminate experiment.

days following inoculation. The animals in the control group received 3 cc. injections of distilled water intraperitoneally on corresponding days. Animals dying obviously as a result of the injections were discarded. The results obtained are given in Table VIII.

Experiment 9.—(Table IX.) This experiment was made up of 7 groups containing 15 guinea pigs each, crossbred in heredity. The organism used in producing the infection was H 37, a human type tubercle bacillus. The animals were inoculated subcutaneously with 1/5 mg. doses. The treatment administered to each group was as follows: 1 group received intraperitoneal injections of 0.1 mg. sodium cholesterylate twice weekly throughout the experiment and commencing 1 week before the time of inoculation. The other 4 groups of treated animals received sodium cholesterylate intraperitoneally on corresponding days but in the following doses: 0.5 mg., 1 mg., 5 mg., and 10 mg. The remaining 2 of the 7

TABLE IX.

Days of Survival Following Infection.

Controls Distilled H_2O	Controls No injections	0.1 mg. sodium cholesteryl-ate	0.5 mg. sodium cholesteryl-ate	1 mg. sodium cholesteryl-ate	5 mg. sodium cholesteryl-ate	10 mg. sodium cholesteryl-ate
49	97	61	67	116	51	60
82	106	80	131	138	51	68
89	114	80	132	140	82	68
96	122	101	137	142	89	70
96	123	103	157	143	97	72
98	132	107	157	145	98	129
101	158	110	166	262	112	164
101	224	167	167	266	162	212
130	236	168	170	266	167	235
146	255	182	175	278	183	
174	258	184	184	326	184	
228	265	286	321	342	196	
244	321	346*	346*	346*	346*	
255	346*	346*	346*	346*	346*	
265	346*			346*		
Average: 143.6	206.9	165.8	189.7	240.1	154.5	119.7

* Killed on 346th day to terminate experiment.

groups of guinea pigs were maintained as controls. 1 of these control groups received intraperitoneal injections of 1 cc. of distilled water on days corresponding to the injections of sodium cholesterylate. The other group received nothing, in the way of injections, after they had been inoculated. All were maintained on a diet of 30 gm. of cabbage per animal per day with hay, oats, and water *ad lib.* Animals obviously dying as the result of the injections were discarded. This eliminated 1 animal apiece from the groups receiving 0.1, 0.5, and 5 mg. sodium cholesterylate. 10 mg. of sodium cholesterylate was obviously an overdose and 6 of the 15 animals in that group died as a result of the injections. The other 9 in

that group then had the apparent toxicity of the large overdose of sodium cholesterylate to deal with in addition to their tuberculosis. The results of this experiment are given in Table IX.

Summary of Results Obtained in Therapeutic Experiments.
A. Results with Cholesterol.

In Experiments 1 and 2, cholesterol definitely prolonged the lives of the animals receiving it. When the significance of these results (8) was determined mathematically it was found that, in Experiment 1, $P = 0.05$.** In Experiment 2 the value of P for the group receiving cholesterol was 0.01 and for the group receiving irradiated cholesterol was 0.07. In these two experiments the infection was of an acute type, the culture very virulent, and the number of infecting organisms relatively small.

In Experiment 3, contrary to what might perhaps have been expected of a chronic infection, cholesterol proved ineffective, the value for P being 0.65. The less resistant animals in the experiment perhaps responded to the treatment with cholesterol, since, in comparing the two series of animals in Table III, it will be noted that among the first animals to die in each series those treated with cholesterol quite definitely outlived the control animals dying in corresponding order.

In Experiment 4 the animals receiving cholesterol tended to outlive the control animals. The margin of advantage shown in Table IV, however, is not mathematically significant since for the group receiving Pfanstiehl cholesterol (hog brain) $P = 0.35$, while for the group receiving gall stone cholesterol $P = 0.60$. Clearly then, the apparent advantage is of no significance. The infection in this experiment proved to be more rapid than in the preceding one, but it was still not the same type of acute infection as in the first two experiments. Whereas, in the first two experiments, the infection was one with a

**In calculating the significance of the results obtained, P is a measure of probability. A value of $P = 0.03$ indicates that the results are significant mathematically and means that the chances are 33 to 1 that the experiment can be repeated with a similar outcome. Values above 0.03 are considered to indicate that the results are not mathematically significant. Values for P below 0.03 are considered very significant as, for instance, $P = 0.01$ where the chances of being able to repeat the experiment are 100 to 1.

very small dose of very virulent organisms, here we had an overwhelming infection with only a moderately virulent culture. Experiment 4 demonstrated, as would be expected, that there is very little difference in the action of cholesterol from different sources.

In Experiment 5, in which a bovine type of infection was used, cholesterol was ineffective, *P* being 0.9.

B. Results with Compounds Containing Cholesterol or Derivatives of Cholesterol.

In Experiment 6, while cholesteryl chloride, toluide, and anilide gave some apparent prolongation of the lives of the guinea pigs treated, the results do not prove to be mathematically significant since the *P* for cholesteryl chloride is 0.1, for cholesteryl toluide 0.25, and for cholesteryl anilide 0.41. The degree of action in each group was roughly proportional to the amount of cholesterol received with each compound.

In Experiment 7 sodium cholesterol sulfate did not significantly prolong the lives of the animals treated, *P* in this case being 0.28.

In Experiment 8 quinine cholesterylate was ineffective in prolonging the lives of guinea pigs treated with it. *P* in this case was 0.65.

In Experiment 9 sodium cholesterylate, in doses ranging from 0.1 mg. to 10 mg., was used in treating guinea pigs experimentally infected with *B. tuberculosis*. Scattered experiments on small numbers of animals had previously given some indication that sodium cholesterylate exerted a favorable influence on tuberculous guinea pigs, several animals outliving untreated animals by long periods of time and showing very little tuberculosis when they were killed at the termination of the experiments. The range of doses used was chosen in the hope of finding an optimum at which the compound was most effective. Two groups of untreated guinea pigs were included. One of these, the true control group, received the same amount of handling and incidental trauma from intraperitoneal injections of distilled water as did the treated animals. The other group of untreated animals was included to obtain some data concerning the actual effect on tuberculous guinea pigs of repeated handling and trauma of injection. They were undisturbed except for weighing and cage cleaning after the time of inoculation. As might perhaps have been expected they outlived

the true control group. Their advantage in days lived following inoculation was significant as P was equal to 0.03. The animals receiving 0.1 mg. sodium cholesterylate showed a somewhat greater average length of life than did the true control group but the difference was not significant as P equaled 0.5. The animals receiving 0.5 mg. sodium cholesterylate showed a still greater prolongation of life than the controls but it was not of significance mathematically as P equaled 0.15. The animals receiving sodium cholesterylate in 1 mg. doses showed an average length of life greater than any of the other groups. The prolongation of life in this group was very significant mathematically as P equaled less than 0.01. The two larger doses of sodium cholesterylate used were obviously too toxic and the animals in these groups had the toxicity of the therapeutic agent to deal with in addition to their tuberculous infection. For the group receiving 5 mg. injections of sodium cholesterylate P equaled 0.82, and for the group receiving 10 mg. injections no calculation was made.

The group of animals receiving 1 mg. injections of sodium cholesterylate appeared to outlive the group of animals that had received no injections or were not handled. The difference, however, was not mathematically significant as P in this case equaled 0.35.

I am very grateful to Dr. John W. Gowen for having calculated the mathematical significance of the results obtained in these experiments. Without his concise evaluation of the data obtained accurate interpretation or analysis of the findings would have been impossible.

DISCUSSION.

Four types of experimental tuberculosis in guinea pigs have been treated with cholesterol administered intraperitoneally. In two experiments, in which an acute type of tuberculosis, due to a relatively small number of very virulent organisms, was treated, the animals receiving cholesterol were definitely benefited. They outlived their untreated control animals by a mathematically significant margin. Cholesterol, however, failed to prolong significantly the lives of guinea pigs exhibiting the other three types of tuberculosis treated. It did not benefit animals having a chronic type of tuberculosis produced by inoculation with a relatively small dose of organisms of low virulence, or those having a more acute type of tuberculosis produced by inocula-

tion with a large dose of organisms of low virulence. It did not prolong the lives of animals infected with a quite virulent strain of bovine type tubercle bacilli.

No explanation of the mode of action of intraperitoneally administered cholesterol can be offered as a result of the work nor is any explanation apparent for the differing results with different types of tuberculous infection. The course of the experiments, and particularly the results of the postmortem examinations give no ground for assuming any direct bactericidal action for cholesterol or any direct and pronounced effect in promoting the healing processes. The connective tissue present in and about the lesions was no greater in treated than in untreated animals, and scars or calcified areas or other evidences of healing processes were no more in evidence. The lymph nodes showed no tendency to regressive changes. Lesions of the usual character prevailed and finally caused the death of most of the animals.

The results with compounds containing cholesterol or with derivatives of cholesterol were, as a whole, quite unpromising. Cholesteryl chloride, cholesteryl toluide, cholesteryl anilide, sodium cholesterol sulfate, and quinine cholesterylate did not benefit tuberculous guinea pigs to a significant degree. Sodium cholesterylate, however, in certain doses proved to be quite effective. 1 mg. given twice weekly very significantly prolonged the lives of the animals receiving it whereas smaller or larger amounts that were tried were less effective. In the case of sodium cholesterylate in optimum dosage, the duration of life was little greater in the animals receiving it than in animals bearing a similar infection but not subjected to any handling or injection trauma. However, a comparison of this sort is unfair for, although sodium cholesterylate failed to do much more than "nothing at all" it very markedly prolonged the lives of animals receiving it when compared to control animals undergoing precisely the same inevitable trauma and handling. The mode of action of sodium cholesterylate is equally as obscure as that of cholesterol.

It is conceivable that the results attained with sodium cholesterylate in optimal dosage may give a starting point for the production of other compounds more effective in the treatment of tuberculosis. Experiments with this view in mind are in progress at the present time.

SUMMARY.

1. Cholesterol, administered intraperitoneally, in these experiments definitely prolonged the lives of tuberculous guinea pigs when the infection was of an acute type produced by inoculation with a small dose of very virulent human type organisms.

2. Intraperitoneally administered cholesterol did not definitely prolong the lives of tuberculous guinea pigs when the infection was of the chronic type produced by the injection of a small dose of human type tubercle bacilli of relatively low virulence, or when the infection was more acute owing to the injection of a large dose of organisms of low virulence. It had no beneficial effect on an acute type of infection produced by the bovine type organism.

3. Cholesteryl chloride, cholesteryl toluide, cholesteryl anilide, sodium cholesterol sulfate, and quinine cholesterylate did not significantly prolong the lives of tuberculous guinea pigs.

4. Sodium cholesterylate, in optimal dosage, definitely prolonged the lives of tuberculous guinea pigs.

5. There was a significant shortening in the duration of life of tuberculous guinea pigs subjected to the trauma of intraperitoneal injection and repeated handling as compared with tuberculous guinea pigs that were not handled or traumatized by intraperitoneal injections.

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CHEMICAL CHANGES IN THE BLOOD OF THE DOG IN EXPERIMENTAL PERITONITIS.

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In previous publications, we have recorded the chemical changes in the blood occurring in pyloric and intestinal obstruction (1, 2). Since distension of the intestine and paralytic ileus are often associated with general peritonitis, a study of the blood chemistry has been made for comparison. It has been suggested that the cause of death in general peritonitis may be due to an intoxication from the intestine rather than to the peritoneal infection.

Method.

Dogs were used for the experimental studies. All operations were done under ether anesthesia with aseptic technique. Peritonitis was produced by ligating the appendix with tape. The animals were kept in a warm room in metabolism cages and allowed to drink water *ad libitum*. Blood for chemical analysis was drawn from the jugular vein daily and at times twice daily if the animal appeared quite ill.

The non-protein nitrogen was determined by the method of Folin and Wu (3), the urea nitrogen by the Van Slyke and Cullen (4) modification of the Marshall method and the carbon dioxide-combining power by the method of Van Slyke (5). The chlorides were determined on the tungstic acid filtrate as suggested by Gettler (6).

OBSERVATION.

Normal dogs weighing from 8 to 13 kilos were used. In all cases the appendix was snugly ligated with tape completely blocking the blood supply. General peritonitis was produced in this way in less than 50 per cent of the animals.

In those dogs developing general peritonitis early changes in the blood chemistry were usually noted. There was a fall in the blood chlorides and a rise in the urea and non-protein nitrogen. The rise in

TABLE I.

Blood Findings in Experimental General Peritonitis.

Dog No.	Day after operation	Blood			CO_2-combining power
		Amount per 100 cc.			
		Total non-protein nitrogen	Urea nitrogen	Chlorides	
		mg.	*mg.*	*mg.*	*vol. per cent*
1	0	26.8	16.8	450	43.8
	1	30.6	10.6	400	45.7
	2	91.5	46.8	330	28.7
2	0	25.4	11.2	490	28.7
	1	42.2	11.9	430	34.3
	2	32.6	21.0	410	34.3
	3	52.0	27.3	380	36.2
	4	31.6	23.1	370	45.7
	5	34.9	13.3	420	43.8
	6	27.3	14.0	380	41.9
	7	22.8	11.9	450	43.8
	8	37.5	20.3	450	49.0
	9	34.5	16.8	430	46.6
	10	50.8	26.6	420	43.8
	11	78.0	45.5	400	32.4
	12	258.0	123.3	340	28.7
3	0	27.3	15.4	560	38.1
	1	27.0	18.2	510	38.1
	2	32.3	20.3	470	38.1
	3	71.4	52.5	440	45.7
4	0	28.0	15.4	460	38.1
	1	40.0	18.9	410	40.0
	2	35.3	21.7	360	38.1
5	0	25.2	13.31	480	34.3
	1	24.2	12.61	460	38.1
	2	28.2	16.81	400	40.0
	3	23.5	11.91	400	36.2
	4	20.2	9.81	400	38.1
	5	56.3	31.51	400	27.5
6	0	32.3	13.3	470	34.3
	1	33.0	12.6	410	45.7

TABLE I—*Concluded.*

Dog No.	Day after operation	Blood			CO_2-combining power
		Amount per 100 cc.			
		Total non-protein nitrogen	Urea nitrogen	Chlorides	
		mg.	*mg.*	*mg.*	*vol. per cent*
6	2	47.2	17.5	370	43.8
	3	31.2	15.4	360	52.0
	4	31.8	16.8	380	41.9
	5 a.m.	34.5	17.5	350	14.3
	5 p.m.	45.8	22.4	320	10.5
7	0	28.0	10.2	450	40.0
	1	31.9	14.0	390	40.0
	2	—	51.3	320	—
8	0	23.4	8.4	490	35.3
	1	22.1	8.4	480	29.6
	2 a.m.	53.2	36.8	440	22.1
	2 p.m.	89.8	49.0	370	—

the nitrogenous elements usually started from 1 to 3 days before the death of the animal and increased until death. The carbon dioxide-combining power did not show any constant change. In some instances there was little or no change, in others a slight increase and in still others a decrease (Table I).

In all animals included in this report a well developed general peritonitis throughout the entire abdominal cavity was found at autopsy. The animals lived from 2 to 12 days following the operation with an average length of life of $4\frac{1}{8}$ days.

DISCUSSION.

The changes here noted in the blood chlorides, urea nitrogen and non-protein nitrogen resemble those observed in pyloric and high intestinal obstructions. In those two conditions an alkalosis develops which is not observed in general peritonitis.

Since the clinical manifestations of acute high intestinal obstruction and general peritonitis are strikingly alike and the chemical changes in

the blood are similar, it seems quite probable that the cause of death may be somewhat similar.

CONCLUSION.

1. A study of the blood chlorides, urea and non-protein nitrogen and the carbon dioxide-combining power in experimental general peritonitis is here reported.

2. The similarity between the chemical changes in high intestinal obstruction and general peritonitis is noted. These chemical changes suggest that the cause of death may be, at least in part, the same in the two diseases.

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THE EFFECT OF OXIDATION OF FILTRATES OF A CHICKEN SARCOMA (CHICKEN TUMOR I—ROUS).

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(Received for publication, June 4, 1928.)

In the course of experiments with the Rous chicken sarcoma, results were obtained which suggested that infectivity of the filtrates was rather quickly destroyed by oxidation. Since the time of Rous' original work on the tumor it has been recognized that saline filtrates become inactive relatively soon at incubator temperatures, and the similar phenomenon in "primary cultures" of the tumor was ascribed by Gye (1) to oxidation in his first paper. Since that time, however, due in part to the work of Baker (2) on the effect of trypsin in Rous sarcoma filtrates, Gye has inclined to the belief that this "auto-inactivation" in saline at 37° was due to proteolytic ferments from the tissue acting on a non-living protein specific factor. He has shown (3) that it may be considerably delayed by HCN in small amounts, which he supposes to be due to a poisoning of the enzyme. Hydrocyanic acid in low dilutions, however, is stated by Oppenheimer (4) to have little deleterious influence on proteolytic enzymes, whereas from the work of Warburg and others it is known to inhibit certain oxidative phenomena almost specifically.

A preliminary experiment showed such striking results when free oxygen was excluded from candle filtrates of the tumor that it seemed worth while to study the phenomenon more carefully. There seemed to be some reason to hope that a partial explanation might be offered for the irregular and discordant results obtained by ourselves and other workers in attempts to repeat the experiments described by Gye (1) in support of his theory of tumor etiology. If, for example, it were possible to show that, like certain bacterial enzymes and toxins (5) an oxidative destruction could be reversed by reducing

agents, the relationship of the phenomenon to Gye's experiments would be evident.

EXPERIMENTAL.

Filtrates of healthy, rapidly growing Rous sarcomas were prepared by grinding the tissue with coarse carborundum in a mortar and suspending the ground mass in saline. The suspensions were centrifuged half an hour, filtered through a thin layer of paper pulp, and finally through a Mandler filter. Such filtrates were perfectly clear, and varied from yellow to pink in color depending on the amount of hemorrhage in the tumor.

Experiment I.

A filtrate prepared as described was divided into two parts. To one was added a solution of cysteine (pH 7.6) to give a final concentration of 1–2000, and after mixing it was covered with melted vaseline (6). A trace of methylene blue added to a similarly prepared test lot was decolorized in 3 or 4 minutes, and remained so for several days at 37°. Nothing was added to the second tube, nor was it sealed by vaseline. Both were incubated at 37°, and small portions removed at intervals for inoculation.

		Chick 1	Chick 2
1. Cysteine Mandler filtrate	$2\frac{1}{2}$ hrs. 37°	++++	+++
2. Plain " "		+++	++
3. Cysteine " "	$9\frac{1}{2}$ " 37°	+++	++
4. Plain " "		++	±
5. Cysteine " "	23 " 37°	+++	+
6. Plain " "		−	−

Experiment II.

The experiment was repeated and amplified by including a parallel test of HCN in a dilution of about 1–10,000.

Experiment II.

	1 hr.		2 hrs.		3 hrs.		5 hrs.		22 hrs.	
Chick....	3	4	5	6	7	8	9	10	11	12
1. Plain filtrate.....	++	+++	++	+	+	−	+	++	−	−
2. Filtrate + HCN.....	++	−	+	−	++	+++	++	++	−	−
3. " + cysteine.....	+++	+++	+++	++	+++	+++	+++	++++	++++	++++
4. " + HCN + cysteine	+++	+++	+++	+	+++	++++	+++	++++	+++	−

Experiment III.

This experiment was designed to compare the action of serum with that of anaerobic conditions induced by cysteine, and to test, at the same time, the effect of simple sealing with vaseline, and of an inert protein such as gelatin. Twelve tubes of 5.4 cc. of a Mandler filtrate were prepared, additions of rabbit serum, both fresh and heated to 56° for 20 minutes, saline, 3.0 per cent gelatin, and 0.05 per cent cysteine were made as indicated below. They were then incubated at 37° and portions removed at intervals for testing. Injections of 0.5 cc. were made in each case.

	8 hrs.				24 hrs.				70 hrs.				7 days			
Chick.....	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
Tube																
1. Fresh rabbit serum 0.6 cc.....	+	+			−	−			†	†			−	−		
2. " " " " " sealed with vaseline.....	?	+			−	−							−	−		
3. Fresh rabbit serum 0.6 cc. + cysteine, sealed.....	+++	+++			+++	+++							−	−		
4. Heated rabbit serum 0.6 cc.....	?	±			−	−							−	−		
5. " " " " " sealed.....	+	+			−	−							−	−		
6. " " " " " + cysteine, sealed.....	++	+++			+++	+++							−	−		
7. Saline 0.6 cc.....			−	−			−	−			−	−			−	−
8. " " " sealed.....			−	−			−	−			−	−			−	−
9. " " " + cysteine, sealed.....			++++	+++			++	+++			++	++++			−	−
10. 3% gelatin 0.6 cc.....			−	−			−	−			−	−			−	−
11. " " " " sealed.....			−	−			−	−			−	−			−	−
12. " " " " + cysteine, sealed...			++++	+++			++	+++			+++	++++			−	−

† Both died in 9 days without tumors, and with no evident reason.

While there are some irregularities in the second experiment it is quite clear that (1) a few hours incubation at 37° will render Mandler filtrates of this tumor non-infectious, (2) that a concentration of 1–2000 of cysteine and protection from the air will defer this loss of infectivity for more than 24 hours and occasionally, at least, much longer, and (3) that cysteine is more effective than a dilution of 1–10,000 HCN, which, as a matter of fact, appears to have a slight deleterious action. Other experiments have shown that HCN in this concentration will regularly preserve infectivity of filtrates for some hours, but we have not seen it persist for as long as 24 hours in any experiment.

The possibility now suggested itself that the extreme variability of infectiousness of candle filtrates of this tumor might be due in part to oxidative changes. Great annoyance is caused by these irregularities which may result in completely inactive filtrates over a considerable period of time, and more frequently in isolated cases. The following experiments were carried out.

Experiment IV.

Tissue from a rapidly growing tumor was minced finely and two lots of 4 cc. each ground in glass mortars with carborundum. One lot was then suspended in 200 cc. of a freshly prepared solution of cysteine hydrochloride 1–2000 in saline brought to pH 7.4 with NaOH. Both portions were centrifuged 15 minutes, the cysteine lot after covering with vaseline. Each lot was then filtered through a layer of paper pulp about 5 mm. thick, and then through Mandler candles. The portion containing cysteine was protected as far as possible from the air by vaseline during both filtrations. Although the paper pulp filtrate still contained enough unoxidized cysteine to decolorize methylene blue, the Mandler filtrate did not. That is to say, protection of the solution containing cysteine had been by no means thorough during the two filtrations. The reagent was gradually oxidized, and by the time the candle filtrate was obtained, it had been completely changed. Probably, however, it had spared to a considerable extent the oxidation of other components of the filtrate. The two Mandler filtrates were then tested.

		Chick 29	Chick 30
Plain	Mandler filtrate	+	+
Cysteine	" "	++++	++++

The filtrate in which oxidation had been partially prevented was strikingly more potent than that made in the usual way. Tumors

began to appear on the 8th day, grew rapidly, and killed in both cases on the 22nd day, while the control tumors appeared only after 14 and 20 days respectively, and were both small.

Experiment V.

The same type of experiment was repeated in such a way as to accentuate the differences due to oxidation. Tumor tissue was finely minced and 2 cc. transferred to each of two large test-tubes. A few glass beads were added to each tube and 50 cc. of saline. One tube was stoppered so as to include about 20 cc. air, while to the second, a fresh solution of cysteine, pH 7.4, was added to a final concentration of 1–2000, then completely filled with melted vaseline, and a stopper inserted in such a way that no air bubbles remained. After the vaseline had solidified, both tubes were shaken for ½ hour, fresh air being admitted to the first tube three times. The saline suspensions were then centrifuged, and filtered through Mandler filters without previous paper pulp filtration. The cysteine lot was protected throughout from the air by liquid paraffin, and a test lot of the Mandler filtrate decolorized methylene blue promptly, showing that unoxidized cysteine remained.

		cc.	Chick 31	Chick 32
Cysteine filtrate,		0.5	++++	+++
"	"	0.25	++++	+++
"	"	0.1	++++	+++
Plain	"	0.5	–	–
"	"	0.25	–	–
"	"	0.1	–	–

In this experiment one cannot be certain to what extent the mechanical effect of shaking may have played a part, since this was obviously greater in the tube containing air. The two experiments taken together indicate clearly that a loss in potency takes place during the preparation of filtrates of this tumor which is due to oxidation and may be largely prevented by adding cysteine and protecting from the air. It is therefore probable that variations in potency of filtrates made in the ordinary way are due, at least in part, to this factor.

Without considerable experience with this method it would be hazardous to assume that active filtrates may be obtained invariably by such means, and obviously in carrying out experiments of many kinds it is highly desirable to know that active filtrates are being used. Filtrates of this tumor which become inert within a few hours at 37°

do so only in the course of a few days in the ice box. If exclusion of oxygen will preserve infectivity for a considerable period at 37° it is reasonable to suppose that it would do so for a much longer time at a low temperature. If this proved to be the case, filtrates could be made and tested, then kept in the cold room preserved with cysteine and vaseline, and used 2 or 3 weeks later when the test inoculations had proved them to be infective. This has been tried with a few filtrates and it appears that tumors develop just as quickly after 3 weeks storage at 3–4° as they do in the case of fresh filtrates. How much longer they may be preserved has not yet been determined.

Reversibility of the Change.

Since Neill (5) has shown that many bacterial enzymes and toxins are readily inactivated by oxidation and in some cases, at least, reactivated by strong reducing substances, it is of importance to determine whether or not the same may be true of the Rous sarcoma agent. If such were the case it might go far toward explaining the results obtained by Gye, and also those of Murphy (7) and of Flu (8). The occasional ability of a "culture" of a tumor or of normal tissue to reactivate Gye's "specific factor" might be due simply to the presence of reducing substances from the tissue, and since this factor appears not to have been considered it is obvious that variations would be expected with such factors as area of surface exposed to air, depth of column, time of exposure, degree to which oxidative destruction of the agent had been carried, etc.

The writer has not been able, thus far, to secure clean-cut results. In experiments designed to show such reversibility indications that it may occur have appeared, but in such a way that other factors appear to be involved. We believe, however, that this possibility must be taken into consideration in carrying out more or less complicated experimental procedures with this virus.

CONCLUSIONS.

1. The rapid autoinactivation at 37° of candle filtrates of the Rous Foul Sarcoma I is due in large part to oxidation.

2. Such oxidation may be prevented by a dilution of 1–2000 cysteine hydrochloride brought to pH 7.4 with NaOH.

3. The variability of infectiousness of candle filtrates is due in part at least to oxidative changes which take place during their preparation; and more active extracts may be obtained by preventing these by means of cysteine.

4. For elaborate experiments it is possible to preserve filtrates in the ice box with cysteine and vaseline until test inoculations have established their infectivity.

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THE RELATION OF THE CAPSULAR SUBSTANCE OF B. COLI TO ANTIBODY PRODUCTION.

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In several previous communications[1] an early mutation of certain capsulated strains of *B. coli* was described. The strains were obtained from the ileum of young calves, dying as a result of the multiplication of *B. coli* in the small intestine and the resulting general intoxication or septicemia. The original colony on agar plates sent out lateral expansions or wings much thinner than the original nucleus of the colony. The bacteria making up this secondary growth were without a capsule and about 1/20 to 1/25 the virulence of the nucleus when injected into the peritoneal cavity of guinea pigs. The bacteria of the colony nucleus were agglutinated only in very low dilutions of the serum of cows treated for months with intravenous and subcutaneous injections of living cultures and cultures heated at 62°C. The mutant was readily clumped in this serum in high dilutions. The agglutination of the (a) or original strain differed from that of the mutant or (b) strain in that a coherent mucoid disc formed in the bottom of the agglutination tube up to 1/10 dilution. In higher dilutions no clumping of any kind occurred. The serum from the cows treated with the original or parent strain protected guinea pigs receiving a surely fatal intraperitoneal dose of both original and mutant strains in doses of 0.005 to 0.01 cc. The serum of untreated cows failed to protect in doses up to 2 cc. With exceptions given below the original (a) strain has maintained its characters in agar slants kept at about 40°F., provided drying out was avoided and the transfer made from the bottom of the tube where some condensation water is always kept. The mutant

[1] Smith, T., and associates, *J. Exp. Med.*, 1927, xlvi, 123–166.

(b) likewise has not changed under these conditions. Virulence of (a) as measured by intraperitoneal injection of living bouillon cultures has fallen, since the minimum fatal dose has risen from 0.025 to 0.06 cc. during 3 years. In the same time the minimum fatal dose of (b) has risen from 0.6 cc. to 1.3 cc. For the bouillon cultures the same lot of bouillon was kept in storage and the cultures used after precisely 24 hours incubation. When a new lot of bouillon was used, the minimum fatal dose was again titrated. In all individual tests controls were introduced however. These two forms (a) and (b) derived from the same bacterium in the colony ((b) obtainable without subjecting bacteria to the Porges' or similar treatment) seemed suited for an examination into the protective and agglutinating antibodies following hyperimmunization.

Agglutination of (a) and (b) Strains.—The sera used in these trials were drawn from cows which had received subcutaneous and intravenous injections of *B. coli* at variable, usually 10 day intervals. *Cow A* received injections of *living B. coli* (a) from November, 1925, to July, 1927. During this period there were several gaps of 3 or 4 months during which treatment was omitted. *Cow B* received injections of *B. coli* (a) heated at 62°C., from September, 1926, to July, 1927. *Cow C* was treated in the same manner but with heated cultures of the mutant (b). In all 17 injections were given to *C*, partly intravenous, partly subcutaneous, extending from Dec. 2 to March 30, 1928. *Cow D* represents sera from various untreated cows. The blood was drawn at different stages of the immunization process into large jars, allowed to clot, and the serum drawn off, passed through Berkefeld filters and stored in full bottles at about 40°F. No appreciable degeneration of the antibody content occurred after months of storage. Agglutinins towards the mutant (b) occur in sera of untreated cows. Clumping may be complete in a 1/80 dilution.

During the immunization of the cows agglutinins towards the original strain (a) failed to appear even in 1/10 dilution until at the end of the treatment when in a 1/10 dilution of cow sera A and B a compact, coherent, mucoid disc formed in the bottom of the agglutination tube. In the 1/20 dilution indications of clumping were noted under the microscope in chain formation of a certain number of bacilli. On the other hand the titer of agglutinins towards the (b) form continued to rise from the start. Towards the end of the treatment agglutination was still recognized under the microscope at a dilution of 1/10,240.

The Thread Reaction.—To determine the influence of agglutinins on bacteria during multiplication bouillon plus immune serum was inoculated and incubated. This method was used first by Ledoux-

Lebard[2] and the following year by Pfaundler[3] and called by him the thread reaction. With this method the clumping of (a) or disc formation was observed at a somewhat earlier period of the immunization process than when the usual method was employed. Several points need consideration when this method is used. In the first place bacteria are exposed to the agglutinins during multiplication. In the second place, the few bacteria introduced at the start are exposed to the full strength of the agglutinin. This gradually diminishes as the bacteria multiply and a point is reached when the agglutinin is entirely used up and the bacteria continue multiplying without clumping.

It was thought that by incubating the bacteria in various dilutions of serum in bouillon in the hanging drop some differences between (a) and (b) in the mode of agglutination might be noted. Thus far this has not been realized. Both (a) and (b) in suitably concentrated immune serum-bouillon grow in the form of interlacing chains of bacilli, each colony forming a distinct circular lacework of chains. Only when the agglutinins are exhausted is this form concealed and modified by diffuse growth. It was thought that this method of allowing growth in serum-bouillon might be useful in the absorption of agglutinins and the following experiments were carried out.

Direct and Reciprocal Absorption of (a) and (b) Agglutinogen in (a) Cow Serum.—When a 1/10 dilution of serum in bouillon was inoculated with a loop of Strain (a) and (b) and incubated the following phenomena were observed after 24 hours.

Strain (a) in Immune Serum-Bouillon.—The fluid is nearly clear. In the bottom is a compact disc-like mass, not disrupted by shaking. The fluid is considerably clouded by the shaking.

Strain (a) in Normal Serum-Bouillon.—Heavy clouding without any coherent deposit.

Strain (b) in Immune Serum-Bouillon.—Growth only in bottom of tube. Fluid clear. When shaken, the fluid becomes heavily clouded with flakes. No disc-like coherent mass. The upturned growth soon subsides.

Strain (b) in Normal Serum-Bouillon.—After 24 hours general

[2] Ledoux-Lebard, *Ann. Inst. Pasteur*, 1897, xi, 909.

[3] Pfaundler, M., *Centr. Bakt., 1. Abt.*, 1898, xxiii, 9, 71, 131.

clouding with a lumpy deposit, easily broken up into smaller lumps, and general cloudiness.

In the course of the following 3 or 4 days there was increasing cloudiness in the immune serum-bouillon tube of (a). The gelatinous disc did not disappear. In the (b) tube the fluid remained clear. All growth took place in the bottom. Evidently in (a) the agglutinins were quickly used up and multiplication went on as in ordinary bouillon. After a week or longer the amount of growth in all tubes was much the same. Although it was chiefly a bottom growth in the (b) tubes, shaking produced a heavy turbidity.

To determine the effects of growth on the fate of the agglutinins, cultures in serum-bouillon were passed through Berkefeld filters. About 0.1 per cent dextrose and 10 per cent distilled sterile water were added to the filtrate to make up for losses and to effect a reduction of the pH through the action of the culture on dextrose. Such filtered fluid was inoculated with both (a) and (b) strains with the following outcome in 24 hours.

1. Serum-bouillon a-a showed no coherent disc-like deposit. The fluid was uniformly clouded with slight, easily suspended deposit.
2. Serum-bouillon a-b had only a bottom growth. This was granular and lumpy when suspended after shaking.
3. Serum-bouillon b-a had a firm, disc-like deposit. Fluid still clear.
4. Serum-bouillon b-b had a granular deposit and clear supernatant fluid.

In 48 hours (3) had become clouded. In 6 days (1) had become heavily clouded. No coherent growth in bottom. (2) was finely clouded with some surface growth. Deposit consists of lumps up to $1\frac{1}{2}$ mm. in diameter. Heavily turbid after shaking. (3) had become moderately clouded with persistent disc. (4) liquid still clear. When shaken the fluid cloudy with clumps up to 3 mm. in diameter.

A second successive filtration with the same additions as after the first filtration was carried out, the fluid tubed in 5 cc. amounts and inoculated with (a) and (b) strains. In the b-a-b tubes a deposit appeared but the supernatant fluid remained clear. When the tubes were shaken the clouding was much less pronounced than in the original cultures. Clumping in groups of 10 to 20 rods was demonstrated with the microscope. The a-b-a tubes were uniformly clouded without deposit. A microscopic examination showed no clumping.

A third filtration was carried out and the filtrate inoculated with (b). The b-a-b-b tubes showed growth in the form of a deposit. The fluid remained clear as heretofore. Even after shaking these tubes were clear on the following day.

The successive cultures in filtrates of serum-bouillon brought out the following facts: The (a) agglutinin was removed by the (a) but not by the (b) type. The (b) agglutinin was present in such amounts that three successive growths of (a) or (b) failed to remove it entirely. The (a) agglutinin forms a very cohesive mass of bacteria which is not broken up by repeated and vigorous shaking. In a normal NaOH solution the mass tends to soften and disintegrate and nearly disappear. In 5 per cent acetic acid it shrinks into a tough leathery membrane of much smaller bulk. Tested with the usual agglutinin technique, the b-a-b filtrate produced only a slight clumping of (b) in a dilution of 1/2 which was equivalent to a 1/20 dilution of the serum itself, thus indicating that the (b) agglutinin had been nearly used up. As a further control a normal cow serum was treated in the manner described, *i.e.* diluted with bouillon 1:10 and inoculated with Types (a) and (b). The (a) fluid became heavily clouded with the usual deposit. When shaken the fluid became more heavily turbid, without any clumping. A disc was not formed. The (b) fluid was at first clear with a bottom growth. Later the fluid became clouded as if the agglutinin present had been used up.

The above cultures were passed through Berkefeld filters and after adding 0.1 per cent dextrose inoculated with (a) and (b). (a) became uniformly and heavily clouded. In (b) a heavy deposit of clumps formed and the clouded supernatant fluid contained both clumps and free forms. Even in normal cow serum-bouillon the (b) agglutinins survived one multiplication of the bacilli.

The Direct and Reciprocal Absorption of Protective Antibodies.—The cow sera A and B tested for agglutinins as described above were also used in the following studies to determine their protective value. Cow A treated with living cultures and Cow B treated with heated cultures of (a) furnished sera of nearly the same protective titer. B was slightly higher and was capable of keeping alive guinea pigs receiving the surely fatal dose (usually $1\frac{1}{8}$ to $1\frac{1}{2}$ the minimum fatal dose) in amounts of 0.005 cc. to 0.01 cc.

The following experiment was carried out twice with entirely concordant results. Only the second is given.

20 cc. tubes of bouillon containing 10 per cent by volume of the immune serum of B were prepared. These were inoculated with *B. coli* (a) and (b). For controls a tube not inoculated was carried along with them and in addition a strain of *B. coli* (1085a) with capsule but without any agglutinative relation to (a). After 50 hours incubation the four tubes were refrigerated and filtered next day through separate Berkefeld filters. The filtrates were tested on guinea pigs to determine protective capacity. Each filtrate was mixed with 24 hour bouillon cultures of *B. coli* (a) and injected into the peritoneal cavity of guinea pigs. In the tests the weights of the guinea pigs were maintained between 350 and 375 gm. The bouillon for culturing was the same throughout. The culture dose was about $1\frac{1}{4}$ to $1\frac{1}{2}$ times the surely fatal dose. The outcome of the test was as follows:

The control serum-bouillon (incubated and filtered with the rest) protected in 0.5 cc., not in 0.3 cc. and 0.4 cc. doses[4]

TABLE I.

The Effect of Multiplication of B. coli (a), (b) and x in 10 Per Cent Immune Serum-Bouillon (a).

Serum used	Per cent serum in bouillon	Growth in serum-bouillon	Minimum protecting dose of serum
			cc.
Cow B	(Original undiluted serum)		0.01
"	10	Control	0.05
"	"	*B. coli* (a)	>0.30
"	"	" (b)	0.05
"	"	" x	0.07

The (a) serum-bouillon filtrate did not protect in 1.5, 2 and 3 cc. doses.[4]

The (b) serum-bouillon filtrate protected in 0.5 and 0.6 but not in 0.4 cc. doses.[4]

The control (1085a) serum-bouillon filtrate protected in 0.7 but not in 0.3 and 0.6 cc. doses. In Table I the serum present in the serum-bouillon is given in the last column.

The (a) strain of *B. coli* removed the protective substance so that 3 cc. serum-bouillon failed to lengthen the life period of the guinea pigs. It thus contained less than 1/6 of the protective substance in the control serum-bouillon. The (b) mutant removed practically none, the other strain of *B. coli* a little. This latter strain was nearly as

[4] To determine the actual serum present divide by 10.

virulent as the (a) strain. It will be noticed that the 10 per cent serum-bouillon control lost more or less during incubation and subsequent filtration. The serum in it protected in 0.05 cc. doses whereas the original serum protected in 0.01 cc. doses or less.

The Content of a (b) Antiserum in Agglutinin and Protective Antibody. —The serum of Cow C (treated with heated (b) mutant) agglutinated the mutant completely up to 1/1,280. At 1/10,240 the microscope still showed about one-third of the rods in clumps. Culture (a) was

TABLE II.

Protective Action of Immune (Cow) Serum (b).

Guinea pig No.	Dose of *B. coli*	Dose of serum	Result
	cc.	*cc.*	
1 (control)	0.06 (a)	—	Dies in 6 hrs.
2	0.07 (a)	0.5	" " 9 "
3	0.07 (a)	1.0	" " 9 "
4	1.3 (b)	—	" " 6½ "
5	1.4 (b)	0.01	" " 16 "
6	1.4 (b)	0.02	Lives

TABLE III.

The Effect of Multiplication of B. coli (a) and (b) in 10 Per Cent Immune Serum-Bouillon.

Guinea pig No.	Dose of *B. coli* (b)	Dose of 10 per cent serum-bouillon	Result Dies in	Amount of serum (calculated)
	cc.	*cc.*	*hrs.*	*cc.*
1	1.3	—	9	—
2	1.4	2 (a)	24	0.2
3	1.4	2 (b)	23	0.2
4	1.5	—	14±	—
5	1.5	3 (a)	14±	0.3
6	1.5	3 (b)	14±	0.3

slightly acted on in low dilutions only. Thus in a 1/2 or a 1/4 dilution the microscope showed chains of 4 to 8 rods among free individuals. About one-fourth of all rods were in short chains. No macroscopic agglutination was recognizable however in these or in the higher dilutions.

The protective action of the serum of Cow C was tested against

both the original *B. coli* (a) and its mutant (b). Omitting the numerous tests for determining the minimum fatal doses of both (a) and (b) and the neutralizing dose of serum we present the final tests in Table II.

It will be seen from Table II that the serum of Cow C treated with mutant (b) failed to protect guinea pigs towards (a) in a dose of 1 cc.[5] whereas 0.02 cc. protected against the surely fatal dose of (b). As has been stated, the (a) serum of Cow B protected against the surely fatal dose of both (a) and (b) in the same small amount of 0.01 cc.

To determine the absorptive capacity of (a) and (b) respectively with reference to the (b) immune serum a 10 per cent serum-bouillon was inoculated with (a) and (b), incubated for 46 hours and filtered. The filtrates were tested for residual protective substances on guinea pigs.

It will be seen from Table III that enough antibody had been removed by both (a) and (b) strains so that 0.3 cc. serum did not even retard death. Higher concentrations were not tried because of the bulk of the serum-bouillon filtrate to be injected. Unfortunately a control serum-bouillon was not carried through, but judging from the result given in Table I we may allow the surely protective dose of the serum-bouillon itself to be 5 times 0.02 cc. or 0.1 cc. serum.

DISCUSSION.

The experiments described point to the existence of two agglutinins, one directed towards the capsule and visualized in the mucoid disc-like mass formed during growth in serum-bouillon, the other directed against the naked bacteria represented by mutant (b). The (a) agglutinin is produced *in vivo* with difficulty, the (b) agglutinin readily and abundantly. The (a) antigen produces both (a) and (b) agglutinins, the (b) antigen only (b) agglutinins. The mutant (b) fails to remove (a) agglutinins while multiplying in (a) serum-bouillon whereas (a) does. These actions are visualized in the agglutinin disc which is absent in culture filtrates of serum-bouillon following the growth of (a) but is still present after the growth of (b).

The protective antibodies, as tested on guinea pigs by mixing serum and living culture and injecting into the peritoneal cavity, follow closely the agglutinins. The (a) serum protects against both (a) and

[5] Higher doses not tried.

(b) forms of the homologous *B. coli* strain, the (b) serum only against (b). Both (a) and (b) strains remove or bind about the same amount of protective antibody in the (b) serum of Cow C. The abundance of (b) agglutinins produced by (a) in Cows A and B is shown by the fact that three repeated crops of *B. coli* in serum-bouillon failed to exhaust the fluid of all (b) agglutinin. A similar abundance of (b) agglutinin was produced in Cow C treated only with mutant (b).

In the preparation of antisera both living and heated (62°C.) cultures of the (a) form were of equal value when injected into cows. The few animals used do not permit generalizations, but it appears that the heated cultures were less injurious to the animal and produced antibodies more promptly perhaps because of the much larger numbers of bacilli that could be injected. Attention is called to the fact that *B. coli* is able to multiply abundantly in the first and second serum-bouillon filtrates of its own growth, less so in the third. After each filtration about 0.1 per cent dextrose was added to lower the pH of the culture fluid through the formation of acid by *B. coli*.

The formation of a coherent viscid disc-like mass as a result of agglutination appears to be associated with capsulated bacteria.

Wadsworth and Kirkbride[6] in testing a pneumococcus serum of Type III observed a prompt agglutination in a 1/1 concentration. "Within 15 to 30 minutes . . . . a loose cap had formed similar to those often seen in tests with Type I and Type II." Clumping was seen no higher than in 1/10 dilutions. Coulter[7] in preparing immune rabbit sera against Friedländer strains observed instant coarse flocculation settling down to a compacted disc in bottom of tube in serum dilutions of 1/1 and 1/5. Small and Julianelle[8] observed the same phenomenon in the agglutination of various strains of *B. mucosus capsulatus*. Avery and Morgan[9] describe antipneumococcus sera when acting on the specific soluble substance of Type II as producing in low dilutions a compact disc-like precipitate, in higher dilutions only flocculation.

The difficulty of producing or failure to produce antibodies towards capsulated strains has been observed and commented on by many working with such forms for the past 30 years. It would seem that

[6] Wadsworth, A. B., and Kirkbride, M. B., *J. Exp. Med.*, 1917, xxv, 629.
[7] Coulter, C. B., *J. Exp. Med.*, 1917, xxvi, 763.
[8] Small, J. C., and Julianelle, L. A., *J. Infect. Dis.*, 1923, xxxii, 456.
[9] Avery, O. T., and Morgan, H. J., *J. Exp. Med.*, 1925, xlii, 347.

such antibodies can be produced by using large, resistant animals and continuing the treatment over long periods. It may seem advisable to compare the horse and the cow with respect to this problem.

It has been noted in a previous paper[10] that the immune serum prepared with the *B. coli* strain (a) failed to protect guinea pigs against certain bovine types of *B. coli* and did protect against others but in doses about 50 times larger than that required against the homologous strain. Recently a highly viscid strain of *B. coli* was isolated from the spleen of a presumably normal guinea pig. The strain failed to ferment saccharose, being in this respect like (a). The viscidity was extreme. Cobweb-like threads several feet long were brought out of the culture tube with the loop and had to be burned off. Early mutation did not take place on agar plates. The intraperitoneal minimum fatal dose of a 24 hour bouillon culture was 0.2 cc. 0.25 cc. culture plus 0.5 cc. serum of Cow B (heated (a) culture treatment) protected. In serum dilutions of 1/2 to 1/20 of Cow B the bacilli were clumped into a floating, gelatinous mass. A certain limited relationship between the capsular material of this form and that of the bovine form is thus demonstrated.

The value of relationships based solely on serologic determinations is somewhat impugned by the above results. If (a) and (b) had been isolated independently, (b) would have been regarded as different from (a) since (b) serum fails to agglutinate (a), to absorb (a) agglutinins and to protect animals against (a). On the other hand (a) serum protects against (b) and agglutinates (b) in high dilutions. Such serologic relationships of bacteria morphologically and culturally alike but obtained from different sources may be regarded provisionally as indicating mutation or degradation of one type or strain (mutant) from the other (original). The taxonomic value of agglutinins obviously must depend on what is agglutinated. The capsular material, being probably developed by parasitism, is the last character to be acquired and most easily lost, as is evidenced by the rapid mutation on agar plates, during which process the capsule disappears. Since the fermentation reactions of (a) and (b) are not changed, it seems reasonable to regard them as of more fundamental value in

[10] Smith, T., *J. Exp. Med.*, 1927, xlvi, 141.

this group in classification than what is denominated the type-specific substance, which seems to be related to or identical with the capsular substance.

It has been stated in a previous paper[11] that immune cow serum does not exercise any appreciable influence on the toxic effect of culture filtrates of (a) and (b) when mixed with them and injected into the jugular vein of calves. It would have been of interest to determine quantitatively if any toxin-neutralizing action could be attributed to the sera of highly immunized cows. Owing to the difficulty of obtaining calves in sufficient numbers and of the same breed at any one time, no accurate comparative quantitative toxin-neutralizing experiments have been made.

CONCLUSION.

The relation between a strain of *B. coli* and its mutant with reference to the production of agglutinins and protective antibodies may be expressed by the statement that the original strain when injected into cows develops antibodies both towards itself and the mutant whereas the mutant produces them only towards itself. The results point to the capsular substance as the material carrying virulence or, expressed somewhat differently, the factor which protects the microorganism in the host.

[11] Smith, T., and Little R. B., *J. Exp. Med.*, 1927, xlvi, 123.

RECIPROCAL EFFECTS OF CONCOMITANT INFECTIONS.

III. The Influence of Vaccinia and of Vaccinal Immunity on the Reaction to Infection with Experimental Syphilis (Intracutaneous Inoculation).

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In a previous paper of this series (1), the results of experiments were reported in which it was shown that in rabbits the efficiency of the reaction to infection with syphilis was markedly decreased by the intracutaneous inoculation of vaccine virus at the time of the intratesticular inoculation of *Treponema pallidum*, the ensuing syphilis being much more severe than in control animals. In other experiments (2), a state of vaccinal immunity at the time of syphilitic inoculation was associated with an increased effectiveness of reaction, the disease being very mild. Results of a similar nature were obtained in which the intratesticular inocula consisted of a mixture of *Treponema pallidum* and vaccine virus (2).

In all the experiments already reported, the syphilitic inoculation was made in one testicle. Our experience, as well as that of others with experimental syphilis, has shown that the circumstances under which the syphilitic infection is initiated are essential factors in determining the general character of the reaction and that, among these factors, the site and mode of inoculation are of particular importance. As an extension of the present study of concomitant infections, experiments were carried out in which another route of syphilitic inoculation was employed, namely, the intracutaneous. In one group of rabbits, vaccine virus was injected intracutaneously at the time of the *pallidum* inoculation but at a different site, while in a second, the animals were immune to vaccine virus when inoculated with syphilis. The results of the experiment comprising the study of the reaction to syphilis induced by intracutaneous inoculation under these two conditions are

reported in detail in the present paper; a preliminary report has previously been published (3).

Materials and Method.

As in the other experiments dealing with the effects of a concomitant vaccinal infection upon the syphilitic reaction, the Nichols strain of *Treponema pallidum* and the Noguchi strain of vaccine virus were employed. The experiment was begun on Feb. 14, 1927, and the materials used for inoculation were the same as in the other groups of rabbits inoculated intratesticularly on this date (1, 2). The syphilitic emulsion prepared from an actively developing testicular lesion contained from 1 to 3 motile spirochetes per microscopic field. The vaccinal material was obtained from an early vaccinal orchitis, and its activity was controlled by the intracutaneous and the intratesticular inoculation of 4 normal rabbits.

The experiment comprised 3 groups of 10 rabbits each. The first group was inoculated with 0.2 cc. of the syphilitic emulsion intracutaneously on the dorsal surface of the prepuce, and immediately thereafter, the vaccinal emulsion (0.2 cc.) was injected intracutaneously on the side of the body while a similar amount was applied to an adjacent scarified area. The skin areas had been shaved just before these procedures were carried out.

The second group of rabbits was immunized to vaccine virus 30 days before inoculation with syphilis. Each animal reacted in a typical manner to the intracutaneous vaccinal injection and a second injection, 10 days after the first, was followed by no visible reaction. The syphilitic inoculation of these animals consisted in the injection of 0.2 cc. of the *pallidum* emulsion intracutaneously on the dorsum of the prepuce, as in the first group of animals.

The third group of the experiment comprised the syphilitic controls, that is, normal rabbits inoculated in the same site and with a similar amount of the *pallidum* emulsion as in the first and second groups.

The rabbits employed were young adult male animals approximately 8 months of age, and were carefully matched as to breed and color. They were separately caged throughout the experiment and were fed the usual diet of hay, oats, and cabbage.

The period of observation was 4 months during which time each rabbit was examined at frequent intervals in order that the course of the disease might be carefully followed. The syphilitic reaction was studied upon the same basis as in the former experiments already referred to (1, 2). Among the significant phenomena of the infection especially considered were the following: the incubation period, the character and duration of the primary lesion, the incidence, time of appearance, number, character, and persistence of generalized lesions, and finally, the resolution and healing of lesions or the initiation of the period of latency.

Although lesions of the testicles, tunics, epididymis, and vas deferens are, strictly speaking, generalized manifestations under the conditions of intracutaneous inoculation, they are of a somewhat different order than lesions of the perios-

teum, bone, skin and mucous membranes, and the eyes, and consequently have been separately considered. As has been found with the intratesticular route of inoculation, the testicles offer extremely favorable conditions for the growth of spirochetes, and in certain rabbits inoculated intracutaneously an orchitis may be the only generalized manifestation detected. Furthermore, no distinction has been made in the present discussion between lesions of the testicles and those of the tunics, epididymis, and vas since the latter structures are frequently involved in an extension of the testicular process.

In analyzing the results, special attention has been paid to the time relations of the successive phenomena of reaction. The term "focal distribution" or "focal rate" as applied to generalized lesions, refers to the number of such lesions per rabbit as determined by clinical examination. The actual rate includes only those animals which developed generalized lesions, while the relative rate refers to all the animals of a group. In estimating the distribution of lesions for group comparisons, the relative rate gives a fairer impression of the extent of the lesions and furthermore, avoids the impression which would be obtained by the chance inclusion in any group of an individual animal with unusually severe syphilis.

RESULTS.

The results of this experiment are summarized in Tables I to III and in Text-figs. 1 and 2. The figures given in Tables I and II represent group values. The incidence of the various phenomena of the syphilitic infection chosen for comparison are given in Table I, together with the actual and relative focal distribution rates of generalized lesions. Table II contains the mean time of occurrence of these phenomena, while Table III lists the time of appearance of all generalized lesions in 2 day intervals as estimated from the date of inoculation. The course of the infection in the 3 groups of rabbits, that is, the animals inoculated with *Treponema pallidum* and vaccine virus, the vaccine-immunes, and the controls is illustrated graphically by the curves of Text-fig. 1 in which the abscissæ represent the animal incidence of the syphilitic phenomena and the ordinates the time in days from the date of inoculation. These curves bring out a characteristic feature of experimental syphilis, namely, that while the events of the infection are successive, they also to some extent overlap each other. The curves of Text-fig. 2 which represent the appearance of generalized lesions in 6 day intervals are compiled from the figures in Table III; an interval of this length was chosen in order to avoid the irregularities of very short periods such as are given in the table.

TABLE I.

Incidence of Various Phenomena of the Syphilitic Infection and the Focal Distribution of Generalized Lesions.

Animal group	Number of rabbits	Chancre	Generalized lesions		
			Incidence	Focal distribution	Focal distribution
		per cent	*per cent*	*actual*	*relative*
Control	10	100.0	50.0	4.4	2.2
V. V.	10*	100.0	88.8	6.0	5.3
V. V. Im.	10	100.0	30.0	1.0	0.3

In this and other tables:

C = syphilitic controls.

V. V. = rabbits inoculated with vaccine virus.

V. V. Im. = rabbits immune to vaccine virus.

* One animal in this group which died of an intercurrent infection 98 days after inoculation has been included in the analysis of the primary but not of the generalized lesions.

TABLE II.

Mean Time of Occurrence of the Various Phenomena of the Syphilitic Infection as Estimated in Days from the Date of Inoculation.

Animal group	Chancre	Generalized lesions					
		First All lesions	First Not testicular	First Testicular	Last	Mean of all	Duration Active period
	days	*days*	*days*	*days*	*days*	*days*	*days*
Control	27.1	75.2	78.0	94.0	101.2	88.4	26.0
V. V.	17.4*	68.1	67.1	72.5	92.6	75.3	24.5
V. V. Im.	37.8	85.3†	75.0	106.0	85.3†	85.3†	0

* The omission from this calculation of the animal which died 98 days after inoculation would diminish the mean value for this group to 16.0 days.

† Since only 1 lesion developed in each of 3 vaccine-immune rabbits, the mean values for the time of appearance of the first and last lesions as well as the mean time of all lesions are identical.

TABLE III.

Time of Appearance of the Generalized Lesions as Estimated from the Date of Inoculation.

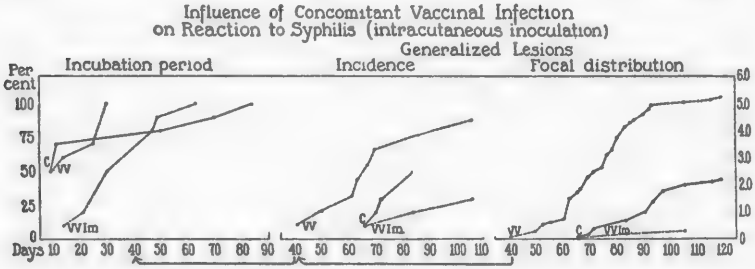
Time interval	Number of lesions			Time interval (cont.)	Number of lesions (cont.)		
	Controls	Vaccinated	Vaccine-immunes		Controls	Vaccinated	Vaccine-immunes
days				*days*			
40	0	1	0	82	0	0	0
42	0	0	0	84	3	4	1
44	0	0	0	86	0	1	0
46	0	0	0	88	0	1	0
48	0	0	0	90	3	1	0
50	0	2	0	92	0	2	0
52	0	2	0	94	4	2	0
54	0	0	0	96	0	0	0
56	0	1	0	98	4	0	0
58	0	0	0	100	0	0	0
60	0	1	0	102	1	0	0
62	0	7	0	104	0	0	0
64	0	0	0	106	1	1	1
66	1	3	1	108	0	0	0
68	0	0	0	110	0	0	0
70	1	4	0	112	0	0	0
72	2	2	0	114	0	0	0
74	0	1	0	116	1	1	0
76	0	4	0	118	0	0	0
78	0	2	0	120	1	1	0
80	0	4	0				
				Total....	22	48	3

DISCUSSION.

In the analysis and discussion of the results of this experiment, the various phenomena of the syphilitic infection have been taken up in the order of their occurrence. The disease picture is thereby simplified and variations in the behavior of the vaccinated and vaccine-immune animals as compared with the controls are the more readily appreciated.

There are certain features of the disease induced by intracutaneous inoculation that should be kept in mind not only in analyzing the present results, but in comparing them with those obtained in other experiments in which the intratesticular route of inoculation was

employed (1, 2). In general, it may be said that the disease as a whole is less certain, and one must take into account the fact that the manifestations of the infection as regards incidence, number, and time relationships are more variable than is the case with intratesticular inoculations. The questions of comparative amounts of inocula, the inherent differences of the inoculated tissues as affecting the growth, multiplication, and dissemination of spirochetes, as well as the more obscure differences of immune reactions that may be presumed to exist in such organs as the skin and the testicle are some of the factors to be considered in a comprehensive comparison of the reaction to infection induced by these two routes of inoculation. It will suffice



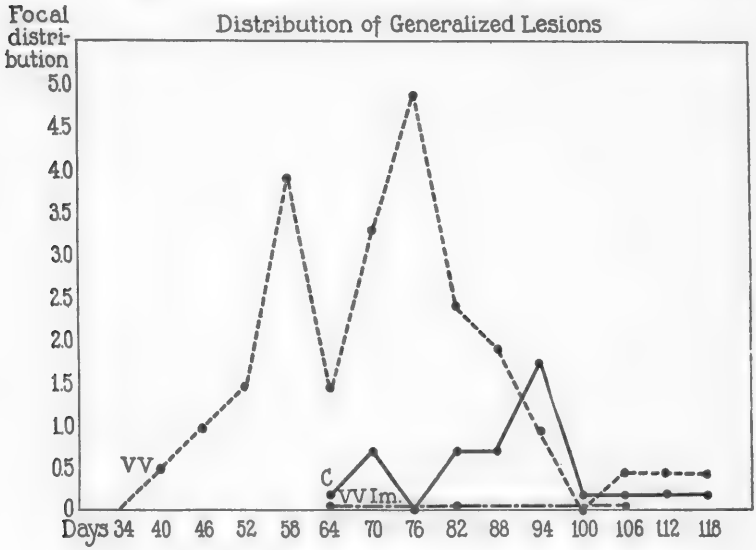
TEXT-FIG. 1.

for the purpose of the present discussion, however, to indicate briefly some of the more important differences in the disease under these conditions.

With an infection of average severity induced by sheath inoculation, the incubation period of the chancre in the majority of a group of 5 or 10 animals is about the same as that of an orchitis, or it may be slightly shorter, owing possibly to the fact that the initiation of a cutaneous reaction is more easily recognized. But there are usually certain animals in which the development of the chancre is considerably delayed and this irregularity is more pronounced both as regards incidence and time than is the case with the testicular primary reaction in a group of rabbits of similar size. Furthermore, the chancre is prone to relapse, and while this tendency also occurs with testicular lesions, it is more frequent and usually more marked. The incidence of generalized lesions is generally lower with the intracutaneous than

with the intratesticular route of inoculation, there are fewer lesions, and the time of their appearance is delayed. It should also be noted that the tendency toward the persistency of lesions noted in connection with the chancre may extend to the secondary manifestations as well.

Chancre.—All animals in each group developed a primary lesion, as shown in Table I. As far as the majority of animals were concerned, there was no difference in the incubation periods of the vaccinated and control rabbits (Text-fig. 1). In 70.0 per cent of the controls, the



TEXT-FIG. 2.

chancres developed in the mean time of 9.6 days while in 60.0 per cent of the vaccinated animals, the mean time was 9.8 days, or 12.0 days in the case of 70.0 per cent of the vaccinated rabbits. But with the remaining animals in each group the mean incubation time was 68.0 days for the 3 controls as contrasted with 28.8 days for the 4 vaccinated animals or 30.0 days if 3 vaccinated animals are considered. If the comparison of incubation time is made from the group standpoint, therefore, the development of primary lesions was more uniform in the vaccinated animals than in the controls. This significant fact is

illustrated graphically by the curves in Text-fig. 1. The mean incubation values reflect the time differences in chancre development of the minority of the animals in each group, the figures being 27.1 days for the controls and 17.4 days for the vaccinated group, a difference of 35.8 per cent.

On the other hand, the primary incubation period was prolonged to 37.8 days in the case of the vaccine-immune animals, or 10.7 days longer than with the controls, a difference amounting to 39.5 per cent. The curve for the vaccine-immune group in Text-fig. 1 representing the initial stage of the disease, however, is again more regular than the curve for the controls, and the position of its last portion in advance of the curve for the controls suggests that in some instances the initial reactivity or sensitivity of rabbits to the intracutaneous inoculation of *Treponema pallidum* may be increased by an immunity to vaccine virus as was also suggested by the results obtained with the intratesticular route of inoculation (2).

Generalized Lesions.—The incidence of lesions which developed at sites other than that of the inoculation area is shown in Table I. Among the controls, generalized lesions were detected in 50.0 per cent of the animals, but the incidence in the vaccinated group was much higher, that is, 88.8 per cent, while it was only 30.0 per cent among the vaccine-immunes.

The distribution of generalized lesions in the three groups was in accord with their incidence as shown by both the actual and relative focal rates (Table II). As compared with the controls, the rates for the vaccinated groups were higher and for the vaccine-immunes much lower. The percentage values of the actual and relative rates of the experimental groups in terms of the control figures bring out clearly the extent of these differences.

Distribution Rates of Generalized Lesions.

Group	Actual	Per cent of control value	Relative	Per cent of control value
Controls	4.4		2.2	
V. V.	6.0	+36.4	5.3	+140.9
V. V. Im.	1.0	−77.3	0.3	−86.4

The generalized manifestations may further be compared upon a time basis, the values for which are given in Table II. As far as the mean time of appearance of the first secondary lesions is concerned, the vaccinated groups antedated the controls by 7.1 days while there was a delay of 10.1 days beyond the control value in the case of the vaccine-immune animals. If the comparison be made with the values of all generalized lesions except those of the testicle, the result is practically the same but the differences are somewhat greater in the case of the testicular lesions alone (Table II), that is, 21.5 days earlier than the controls for the vaccinated group and 12.0 days later than the controls for the vaccine-immune animals. The most interesting point in regard to the first testicular lesions, however, is that they occurred at about the same mean time as the other initial secondary manifestations in the vaccinated group, while they developed much later in the controls and the vaccine-immune animals. Thus, differences in the time of appearance of the first testicular lesions and of other generalized lesions as compiled from the values given in Table II are: vaccinated group, 5.4 days; controls, 16.0 days; vaccine-immune group, 31.0 days.

In regard to the mean time of appearance of the last generalized lesions, the largest value occurred with the control group, that is, 101.2 days as compared with 92.6 and 85.3 days for the vaccinated and vaccine-immune groups respectively. But it must be remembered that these figures are mean values, and reference to Text-fig. 1 shows that generalized lesions continued to appear in the vaccinated group toward the end of the observation period as they did in the controls. In the case of the immune rabbits, however, only 3 generalized manifestations were detected, 1 each in 3 animals, so that the mean values for the appearance of the first and last lesions are identical. It should be noted, however, that the last of these lesions to develop appeared 14 days before the last lesions observed in the other 2 groups as shown by the curves in Text-fig. 1.

The duration of the active period of generalized lesions as calculated from the mean values of the time of appearance of the first and last lesions was practically the same for the controls and the vaccinated groups, 26.0 and 24.5 days respectively, but with the vaccine-immunes,

the period of eruptive activity cannot be calculated on this basis, for as has just been mentioned, the 3 lesions in this group developed in 3 different animals (Table II, Text-fig. 1).

As far as the mean time of appearance of *all* generalized lesions is concerned, the value for the vaccinated rabbits is smaller than for the controls, that is, 75.3 and 88.4 days respectively (Table II), which suggests that a large number of lesions in the former group developed at an earlier period. That this was actually the case is shown by the distribution curves of Text-fig. 2 in which the appearance of lesions is plotted upon a time basis. In drawing these curves, the relative focal rates of generalized lesions have been used, so that one also obtains an idea of the extent of the eruptive period in the 3 groups of animals. It is at once evident by comparing the shape and height of the curves for the vaccinated and control groups that a large number of secondary manifestations had developed in the vaccinated animals before any were detected among the controls, indicating a more prompt and vigorous reaction on the part of these rabbits as, it will be remembered, was also the case with the primary lesion. Furthermore, the height of eruptive activity in the vaccinated group occurred from 70 to 82 days after inoculation, while with the controls it was from 82 to 94 days. These differences account for the magnitude of the values for the mean time of appearance of all generalized lesions.

An interesting feature of the general character of syphilitic infections is illustrated by these curves. The development of many generalized lesions is frequently followed by a more or less prompt cessation of such activity, while if fewer lesions are distributed over a similar or somewhat longer period, the phase of eruptive activity is apt to continue for some time. Among the control animals of this experiment, for instance, secondary lesions first appeared 64 days after inoculation, and they continued to develop at approximately the same rate until the 100th day and from then on at a much lower rate to the 120th day when the observations were discontinued.

With the vaccinated group, on the other hand, the first generalized lesions appeared on the 40th day after inoculation. During the next 18 days a large number of lesions developed, and although this phase was followed by a brief fall in eruptive activity, it was succeeded by a period of 30 days during which a great many lesions developed. In

the last fortnight of the experiment, a few lesions continued to appear, and although the focal rate was much lower than it had previously been, it was definitely higher than that of the control group at this time. There are 3 points in particular in which this course of infection was unusual. In the first place, the initial phase of pronounced eruptive activity commenced unusually early and it continued for a considerable time at a very high rate and thirdly, there was a continuance of activity late in the course of the disease when one might expect a cessation of generalized lesions in view of the preceding course of events. It would appear, therefore, that the reaction of the vaccinated animals to the syphilitic infection was not only affected at the initiation of the phase of secondary manifestations, but for a considerable time thereafter and in an unusual manner.

As far as the vaccine-immune group is concerned, the 3 points indicated in Text-fig. 2 show in contrast to the curves representing the vaccinated and control groups, the very slight extent and degree of the generalized phase of the infection. This feature is also illustrated in Text-fig. 1 by the low height of the curve representing the focal distribution of secondary lesions among the vaccine-immune animals.

In discussing the generalized phase of the infection, the question of the particular site or tissue involved has been mentioned only in connection with the time of appearance of the first generalized lesion, that is, as to whether the site of the first lesion was or was not testicular. The relative incidence of testicular involvement as compared with other secondary lesions is shown in the following table:

Incidence of Generalized Lesions in Different Sites.

Group	Testicle	Bone and periosteum	Skin and mucous membranes	Eyes
	per cent	*per cent*	*per cent*	*per cent*
Control	50.0	40.0	10.0	0
V. V.	55.5	77.7	22.2	0
V. V. Im.	10.0	20.0	0	0

All control rabbits which developed generalized manifestations developed testicular lesions as well, but there were 3 vaccinated and 2 vaccine-immune rabbits which did not. On the other hand, there

were 1 vaccinated and 1 vaccine-immune animal, as there was 1 control, in which a testicular lesion was the only generalized manifestation. These results should be considered in connection with the extent of involvement of the above sites.

Number of Lesions in Different Sites.

Group	Testicle		Bones and periosteum		Skin and mucous membranes	
	No.	Per cent of all lesions	No.	Per cent of all lesions	No.	Per cent of all lesions
Control	6	27.3	15	68.2	1	4.5
V. V.	6	12.5	39	81.2	3	6.3
V. V. Im.	1	33.3	2	66.7		

From these two analyses, it is evident that while the incidence of testicular involvement was the same in the control and vaccinated groups, the latter group showed a far greater number of bone and periosteal lesions. In addition, cutaneous lesions developed in 2 vaccinated as compared with 1 control animal. The relative proportion of testicular and bone lesions in the 2 groups as shown by the percentage values above is a further indication of the disturbed reaction of the vaccinated animals, a disturbance which is both quantitative and qualitative. In the vaccine-immune group, on the other hand, the general type of the reaction as judged by the percentage values of the various kinds of generalized lesions was similar to that of the controls, but the great difference in the actual numbers of lesions shows at once a quantitative difference in the reaction of the vaccine-immune animals.

Recovery.—The last point to be discussed is the condition of the lesions 4 months after inoculation. As with the intratesticular route of inoculation, there is a considerable variation at this time in a group of 5 or 10 rabbits with respect to the proportion of animals in which all lesions have healed, in which case the infection is said to be latent, and the number of animals in which lesions are still present. In this connection, it should be recalled that chancres are especially prone to relapse, and that with the intracutaneous route of inoculation, generalized lesions usually develop later than with an intratesticular inocula-

tion. The final observations in this experiment are summarized in the following tables in which the lesions are classified as healed, not healed, or active.

State of Primary Lesions 4 Months after Inoculation.

Group	Healed	Not healed	Active
	per cent	*per cent*	*per cent*
Control	60.0	30.0	10.0
V. V.	33.3	22.2	44.4
V. V. Im.	60.0	40.0	

State of Generalized Lesions 4 Months after Inoculation.

Group	Healed	Not healed	Active
	per cent	*per cent*	*per cent*
Control	50.0		50.0
V. V.	22.2		77.7
V. V. Im.	90.0		10.0

It is shown by these analyses that active primary and generalized lesions were present 4 months after inoculation in a greater proportion of vaccinated than control animals, while the reverse obtained with the vaccine-immunes. The results are of importance in that they are of the same general order as those already discussed in connection with the earlier phenomena of the syphilitic reaction, but their special significance lies in the fact that the effect of a concomitant vaccination or of a vaccinal immunity was sustained for so long a period.

From what has been said regarding the results of the experiments here reported, it is evident that intracutaneous inoculation with vaccine virus at the time of intracutaneous inoculation with *Treponema pallidum* was associated with a well marked disturbance in the syphilitic reaction, particularly as regards the incidence and time relationships of reactive phenomena, the relative frequency of occurrence of various manifestations, and the relative failure on the part of an early and pronounced generalized phase to reduce or prevent the continuance of eruptive activity. Judging from the comparisons made of the various criteria selected for analysis, it is clear that the efficiency of reaction in these animals was definitely lowered with the result that the infection was severe and prolonged.

With respect to the vaccine-immune rabbits inoculated intracutaneously with *Treponema pallidum*, on the other hand, the incidence and time relationships of reactive phenomena as well as the relative frequency of occurrence of various manifestations demonstrate very definitely that the syphilitic reaction was also affected, but in the direction of increased efficiency with the result that the infection was mild and comparatively short in duration.

Bearing in mind the fact that the syphilitic infection induced by an intracutaneous inoculation differs in several important respects from that resulting from the intratesticular route, the present results are in substantial accord with those previously reported (1, 2). In both instances, vaccine virus injected intracutaneously at the time of syphilitic inoculation was associated with a severe disease and likewise in both instances, a state of vaccinal immunity at the time of syphilitic inoculation was associated with a mild disease. Whatever may prove to be the mechanism by which these effects are brought about, the influence of the two conditions under discussion, that is to say, a concomitant vaccinal infection and a concomitant vaccinal immunity, was sufficiently pronounced to be perceptible in well defined modifications of the syphilis induced by two quite different routes of inoculation.

It is well recognized that the syphilitic reaction may be variously modified by a number of procedures, as for instance by different forms of treatment, surgical measures, and the method and route of inoculation. The results of these and previous experiments already referred to demonstrate that the reaction may also be affected by the presence of another infection as well as by the presence of an immunity to this second infection. It will be recalled, however, that in the case of vaccinia as compared with syphilis, the infection is of short duration and that immunity develops rapidly. It would appear, therefore, that the conditions present at the time of syphilitic inoculation constitute a factor of major importance in determining the course of infection.

Although little is known regarding the nature of the syphilitic reaction, there can be no doubt but that it involves many factors that are essentially expressions of functional activity, and from this standpoint it appears that the influence of a concomitant vaccinal infection or of a preexisting vaccinal immunity is mainly concerned with the

properties of host reaction and resistance. But the information regarding the question of concomitant diseases is meager, and the possibility that conditions affect the spirochetes, either directly or indirectly, must not be forgotten.

SUMMARY.

An experiment is reported in which was studied the effects of a concomitant vaccinal infection and of vaccinal immunity upon the reaction to syphilis in rabbits induced by intracutaneous inoculation.

The results obtained showed that the reaction was modified by both conditions. A vaccinal infection initiated at the time of syphilitic inoculation was associated with a defensive reaction of lessened efficiency, the ensuing syphilis being more severe than in control animals. A state of vaccinal immunity present at the time of syphilitic inoculation was associated with a reaction of heightened efficiency, the ensuing syphilis being very mild.

These results are in harmony with those obtained in other experiments in which the intratesticular route of syphilitic inoculation was employed.

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THE ACTION OF THE LEVADITI STRAIN OF HERPES VIRUS, AND OF VACCINE VIRUS IN THE GUINEA PIG.

Single and Combined Effects.

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The guinea pig is susceptible to most strains of the virus of herpes, but there are certain ones, such as the Levaditi *souche* C, which, on intracerebral inoculation in this animal,[1] are inactive. Flexner[2] has shown that such differences are explainable on the basis of variations in virulence, weak strains being implanted on the cerebrum with difficulty and strong strains with ease. An interesting analogue to this condition may be found in such a widely different infectious agent as the filtrable virus of foot-and-mouth disease. Among types of this virus some may be found which are easily transferred to guinea pigs (Type A, for example),[3] while others are not.

The resistance of the guinea pig to some strains of herpes virus has been recently emphasized by Dmitrieff[4] and Rose and Walthard.[5] They consider that the behavior of the animal in this regard offers a possible solution to the problem of the etiology of epidemic encephalitis in man. Their idea is that the inability to transfer this disease from man to rabbits may be explained by a natural resistance such as that manifested by guinea pigs to herpes virus. Flexner,[2] on the other hand, has brought out the point that the guinea pig merely acts to discriminate strong from weak strains of herpes virus. Consequently the recognition of this factor does not aid in the solution of the etiology of epidemic encephalitis.

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In this paper we intend to present the results of attempts to implant a weak strain of herpes virus on the cerebrum of the guinea pig by the use of special methods. In some of the tests dermo- and neurovaccine viruses were used. The experiments included a study of the nature of the barrier to implantation of weak strains, as well as one of immunity reactions to both weak and strong strains.

In carrying out the following tests, we employed as a sample of a weak strain of herpes virus the Levaditi C strain which, in agreement with previous findings,[2] has failed to infect a large number of guinea pigs after ordinary intracerebral inoculation. In some tests this virus was used by itself, and in others in combination with the viruses of vesicular stomatitis of horses,[3] or of vaccinia, both dermovaccine and neurovaccine.[6] Experiments of the latter sorts involved necessarily a study of the individual effects of the vesicular stomatitis and vaccine viruses in the guinea pig, so that the action of these when combined with the Levaditi strain could be properly understood. For certain purposes, a strong strain of herpes virus was needed. We then used the H.F.[1] and the J.B.[7] strains.

The methods employed in preparing suspensions of brain for inoculation, the manner of injection, and the observations of treated animals followed the procedures of Flexner and Amoss,[7] unless otherwise stated.

Survival of Herpes Virus (Levaditi Strain) in the Brain of the Guinea Pig.

The first series of experiments[8] related to a study of the period of survival of the Levaditi strain of herpes virus in the brain of the guinea pig.

For this purpose, a number of guinea pigs, as shown in Table I, were injected intracerebrally in one hemisphere, with controlled, active Levaditi virus, as contained in the cerebral tissue of rabbits dying from virus encephalitis. The amount of active rabbit brain used for inoculation of the guinea pigs was, as a rule, 0.2 cc. of a 10 per cent saline suspension. After various periods of time, the animals were killed and a fragment of forebrain, usually about 0.5 gm. in weight, was removed in a sterile manner from a region showing the track of the needle. A 10 per cent saline suspension of this fragment was made and 0.4 cc. inoculated intracerebrally into each of two rabbits. The animals were then closely studied for any indication of virus encephalitis. The brains of the rabbits which suc-

[6] For two samples of neurovaccine placed at our disposal, we are indebted for one to Dr. Rivers, of The Rockefeller Institute, who had originally received the strain from Professor Levaditi, and for the other to Professor Levaditi himself.

[7] Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1925, xli, 215.

[8] Ether anesthesia was employed on animals in all experiments.

cumbed, or were etherized when moribund, were sectioned and stained, and examined for the characteristic lesions of infiltrative meningoencephalitis, and for

TABLE I.

Period of Survival of Levaditi Virus in Guinea Pigs' Brains; as Demonstrated by Inoculations of the Brain Tissue into Rabbits.

Series	Days after intracerebral injection of the guinea pigs	Effects on the rabbits
A	2	No reaction
	3	" "
	4	1 of 2 rabbits showed characteristic virus encephalitis; death on 4th day
	5	1 of 2 rabbits showed characteristic virus encephalitis; death on 5th day
	7	No reaction
	9	" "
B	1	2 rabbits showed characteristic virus encephalitis; death on 7th and 9th days respectively
	2	No reaction
	3	" "
	4	" "
	5	2 rabbits showed characteristic virus encephalitis; death on 9th and 11th days respectively
	6	No reaction
C	1	2 rabbits showed characteristic virus encephalitis; death on 5th day in one instance and moribund on 5th day in the other
	2	No reaction
	3	" "
	4	" "
	5	" "
	6*	1 of 2 rabbits showed characteristic virus encephalitis; death on 9th day

* In an additional experiment two guinea pigs from which the cerebral fragments were removed 9 days after inoculation of virus failed to reveal the presence of virus by the rabbit test.

the typical intranuclear inclusion bodies. Cultures of the brains in chopped meat medium and in dextrose broth were also made to rule out the action of bacteria. The results are given in Table I.

It will be noted in the experiments of Series A that the brains of guinea pigs inoculated with the Levaditi virus were not active on the 2nd and 3rd days after cerebral injection, but were active on the 4th and 5th days. Thereafter, to the 9th day after injection, the virus could not be detected by means of the rabbit test. Also in Series B, virus was obtained from the guinea pigs' brains on the 1st and 5th days after injection, but not on the 2nd, 3rd, 4th, and 6th days. In Series C, virus could be demonstrated by the rabbit test on the 1st and 6th days after inoculation, but not on the 2nd to 5th days, and in an additional experiment, not on the 9th day.

One may suppose that the Levaditi strain of herpes virus, after its introduction into the brain of the guinea pig, remained there for 24 hours after inoculation in a concentration sufficient to show activity when the inoculated area was transferred to the cerebrum of the susceptible rabbit. In other words, a passive transfer of the virus might have occurred, explaining the positive results at this time. Thereafter, up to about the 4th day the virus was not detected. During this period, we believe the virus to have scattered throughout the cerebrum, and since it again became demonstrable by the rabbit test on the 4th to 6th day after injection, it probably increased in amount during this time. Thereafter it was not again demonstrable, a fact which would imply its final neutralization or elimination. It is important to note that during all this time the guinea pigs appeared healthy, and those brains removed for injection into rabbits were free from distinctive histopathological changes.

The brain of a guinea pig which had been found to contain virus failed to produce virus encephalitis after 53 days glycerolation, unlike glycerolated rabbit brains in which the virus survives for an indefinite period. From this experiment one might perhaps infer that in the guinea pig brain the concentration of virus was less than in the rabbit brain.

Neutralization Tests with Serum.

The following tests were made to determine whether any neutralizing factor for herpes virus exists in normal guinea pig serum.

Four experiments were planned, all with the same methods.

Preparation of Virus.—The source of the Levaditi herpes virus was fresh cerebral tissue of a rabbit dying from 3 to 7 days after intracranial inoculation of the virus. A 10 per cent saline suspension was made which was filtered without pressure through one layer of filter paper, for the purpose of removing the larger particles. In one test the H.F. strain of virus was similarly prepared and used as a control.

Preparation of Serum.—Two or three full grown guinea pigs were bled by cardiac puncture. The blood was pooled and kept at 37°C. for about an hour until the serum separated from the clot, when it was centrifugalized. In some tests, part of the serum was heated at 56°C. for ½ hour to destroy complement; the remainder was used in a fresh state.

Plan of Test.—As a rule, five tubes were prepared with 0.1 or 0.2 cc. of the paper-filtrate virus. To the first tube, 2 cc. of saline solution was added (control), to the second and third, 2 cc. and 1 cc. of inactivated serum were added, and to the fourth and fifth, 2 cc. and 1 cc. of fresh serum. The mixtures were repeatedly shaken and placed at 37°C. for 1 hour, and then overnight in the ice box at 5°C. Rabbits were then inoculated intracerebrally with 0.35 to 0.4 cc. of each of the mixtures to determine the survival of the virus.

In addition to these experiments, another one was made in which suspensions of normal guinea pig brain were added to the virus instead of the serum.

To summarize the results, suspensions of cerebral tissue or inactivated serum from normal guinea pigs failed to neutralize the virus. With fresh, normal guinea pig serum, however, complete neutralization was effected in one experiment, the incubation period was protracted to 11 and 13 days respectively (as compared with 6 and 7 in the controls) in two others, and no neutralization occurred in the last. In the case of the H. F. strain of herpes virus, to which the guinea pig, as a rule, responds with encephalitis, neither inactivated nor fresh serum neutralized the virus. It may be concluded that the results, although not entirely consistent, suggest that fresh, normal guinea pig serum has a neutralizing action on the Levaditi virus.

Inoculation of Levaditi Virus Alone.

The next series of experiments involved attempts to produce encephalitis in guinea pigs with the Levaditi virus, by various special means. To obtain active virus, we employed fresh, non-glycerolated brains removed from rabbits either in the terminal stages of Levaditi-virus encephalitis or shortly after death.

The first method involved the inoculation into the brain of a large amount of virus (0.05 to 0.1 cc. of a 10 per cent rabbit brain suspension) into three apparently healthy, 1 week old guinea pigs. Young animals were used to ascertain whether they might be less resistant.

The second attempt included the subdural injection of massive doses of the virus, that is, 0.2 cc. of a 10 per cent rabbit brain suspension into each side of the forebrain of normal 250 gm. guinea pigs. It was believed that this saturation of the brain to the limit imposed by the operative technique might yield different results from the usual injection of 0.15 cc. into one hemisphere.

In the third instance, medium-sized guinea pigs were inoculated intraperitoneally with massive doses of Levaditi virus (5 and 10 cc. of the usual fresh brain suspension) and at the same time intracerebrally with the ordinary amount —0.15 cc. In this series the attempt was made to saturate the body with virus and reduce the general resistance.

The fourth test consisted of the intracerebral passage of the virus at 5 day intervals through three successive series of guinea pigs. We aimed at the enhancement of the virulence of Levaditi virus by repeated transfer of cerebral tissue at a period corresponding to the maximum concentration of the virus, as shown in the first set of tests.

In a fifth experiment, we made use of the method of Teague and Goodpasture[9] who reported increase in virulence of herpes virus after its inoculation into the tarred skin of guinea pigs. Seven young, white-furred guinea pigs were subjected to treatment with Holland tar. Acting upon the finding of Teague and Goodpasture, that the sensory nerves and posterior root ganglia corresponding to the area of skin treated are affected, we applied the tar as close to the head as possible. The liquid was painted on the back of the neck and upper posterior chest four times at 3 to 4 day intervals. At the end of the period, the skin was hairless and hypertrophic. 10 per cent saline suspensions of fresh rabbit brain containing active Levaditi virus were then rubbed into the scarified areas, and injected intracutaneously into five of the pigs; the remaining two served as controls. The virus treatment was repeated after 48 hours and again after 5 days.

In the sixth test the Levaditi virus, as contained in rabbit brains, was inoculated into the scarified corneæ of guinea pigs. Suspensions of scrapings of the cornea of these animals were inoculated into normal guinea pigs, in this way propagating the virus through five successive corneal passages, in twenty animals.

None of these six methods served to establish specific encephalitis in guinea pigs. Neither were any local specific lesions manifest in any instance, except in the one in which the virus was inoculated into the cornea. In the series of corneal inoculations, all guinea pigs

[9] Teague, O., and Goodpasture, E. W., *J. Med. Research*, 1923, xliv, 185.

of the first to the fifth passages showed a definite but mild keratoconjunctivitis, from which they recovered without showing cerebral symptoms.

In view of the finding, however, recorded in the first experiment, namely, that the Levaditi virus increases in quantity in the brains of guinea pigs, another mode of definite implantation of the virus was still searched for, and one was found.

The procedure consisted merely in a combination of corneal and subdural inoculation of the Levaditi virus into the same guinea pig at different times.

Two guinea pigs were injected intracerebrally with 0.2 cc. of a 20 per cent saline suspension of fresh rabbit brain containing Levaditi virus. At the same time, the scarified left cornea was inoculated with the same suspension of virus. The next day the animals revealed definite keratoconjunctivitis. On the 3rd day after the first treatment, the animals were inoculated in the scarified right cornea with the corneal scrapings derived from a guinea pig with keratoconjunctivitis resulting from a corneal virus inoculation. On the 4th day both eyes of each animal exhibited keratoconjunctivitis. On the 6th and 7th days respectively, both guinea pigs became hypersensitive and showed circling movements, tremors, involuntary muscular contractions, urine retention, gnashing, and marked salivation. When moribund, they were killed and the brains removed for culture, histopathology, glycerolation, and for further guinea pig passages. In gross the brains were quite normal, except for a slight injection; the other organs were unaffected. Cultures of cerebral tissues gave no growth. Microscopic examination of the brains disclosed the severe, infiltrative meningoencephalitis characteristic of herpes-virus encephalitis in rabbits and guinea pigs. There were also present large numbers of intranuclear inclusion bodies typical of herpetic infection.

This experiment was repeated three times with the same outcome in each.

Thus, finally, characteristic herpes-virus encephalitis was induced in the guinea pig with the Levaditi strain. Moreover, the effects were obtained through seven successive guinea pig passages:[10] the first to the third by the special combination of subdural and corneal inoculations; the remaining passages by intracerebral injections only. On transfer of the suspensions of cerebral tissue to rabbits' corneæ or brains, the latter developed typical herpes-virus effects followed by

[10] After four to five successive passages in the guinea pig, with subdural inoculation only, the experiments were discontinued.

death from encephalitis. Furthermore, after the virus acquired its encephalitogenic property, the usual dose employed with strong strains (the H.F., for example) sufficed to induce the characteristic encephalitis in guinea pigs. Moreover, the encephalitogenic Levaditi virus behaved like strong strains in guinea pigs in that the kerato-conjunctivitis tended often to complete recovery. As is also the case with strong strains injected intracerebrally in guinea pigs, recovery would occasionally occur in an animal of a series which showed the characteristic signs of encephalitis.

Having succeeded in adapting the Levaditi C virus to guinea pigs by the double method of inoculation described, the concomitant effects of the viruses of vesicular stomatitis and of vaccinia were next tested.

Concomitant Effects of Levaditi Virus and Other Viruses.

Recent reports have shown the growth and survival of vaccine virus in tumors of mice and rats (Levaditi and Nicolau[11]), of Virus III, and vaccine virus in a transplantable rabbit neoplasm (Rivers and Pearce[12]), and the enhancement of the effects of syphilis in rabbits with vaccinia (Pearce[13]). Levaditi and Nicolau[14] have reported also on the concomitant action of herpes and vaccine viruses in rabbits, through which vaccine virus was rendered more active and made to induce characteristic encephalitis.

For experimental purposes the virus of vesicular stomatitis of horses, already described[3, 15] and vaccine virus were used. The latter was generously supplied by the New York City Health Department Research Laboratories.

Vesicular Stomatitis Virus.—This material consisted of a fixed guinea pig passage virus, propagated through several hundred animals. It was capable of inducing characteristic vesicles in the pads and corneæ of the animals 24 to 48 hours after inoculation, but was free from neurotropic action.

Vaccine Virus.—The virus consisted of calf lymph that was active in the skin, testicle, and cornea of guinea pigs, producing typical vaccinal lesions within 2 to 4 days after inoculation. This sample of virus was not neurotropic, failing to induce encephalitis after intracerebral inoculation of guinea pigs.

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[11] Levaditi, C., and Nicolau, S., *Ann. Inst. Pasteur*, 1923, xxxvii, 443.
[12] Rivers, T. M., and Pearce, L., *J. Exp. Med.*, 1925, xlii, 523.
[13] Pearce, L., *J. Exp. Med.*, 1928, xlvii, 611.
[14] Levaditi, C., and Nicolau, S., *Compt. rend. Soc. biol.*, 1925, xciii, 3.
[15] Olitsky, P. K., *J. Exp. Med.*, 1927, xlv, 969.

Tests.—Different combinations of viruses were made, as follows: Levaditi C virus was injected intracerebrally in guinea pigs of all the series. In two of the series, vesicular stomatitis and vaccine viruses were also inoculated intracerebrally at the same time. In a third series, vesicular stomatitis virus was injected in the pads at the time the Levaditi virus was introduced into the brain; and in a fourth, vaccine virus was injected intracutaneously. In each instance the viruses by themselves were found to be active; the Levaditi virus in control rabbits, and the vesicular stomatitis and vaccine viruses in control guinea pigs.

The simultaneous injection of Levaditi virus in the brain and vesicular stomatitis or dermatotropic vaccine virus also in the brain, or in the skin, failed, on the other hand, to produce any cerebral effect.

In the next experiments two specimens of neurovaccine were used in additional tests on the combined action of the viruses. The neurovaccine was much more active than the dermatotropic sample in the skin, cornea, and testes of guinea pigs.

Characteristics of Neurovaccine Virus.—The samples of neurovaccine virus were alike in their action in animals.

In rabbits injected intracerebrally, the virus acted in a manner similar to the herpes virus. The symptoms shown by the inoculated animals were identical, except that in the case of neurovaccine virus the experimental disease was more severe; the first signs were manifest within 48 hours after inoculation, and death ensued, as a rule, within 72 to 96 hours. The histopathology of the brains consisted of infiltrative meningoencephalitis, but the characteristic intranuclear inclusion bodies of herpes encephalitis were absent.

The neurovaccine virus differs from herpes virus in the failure to show cross-immunity reactions. Rabbits recovered from keratoconjunctivitis induced by the H.F. II strain of herpes virus[16] were not resistant to later intracerebral injections of neurovaccine virus. Furthermore, the neurovaccine virus produced characteristic vesicular keratitis in rabbits and Guarnieri bodies were found in the lesions. Finally, the virus was active in the skin and testes of rabbits, in which typical vaccinal eruption or the orchitis was produced.

The effects of neurovaccine in guinea pigs differ from those in rabbits to a noteworthy degree. After intratesticular injection an orchitis developed on the 2nd day which tended to heal rapidly. No cerebral involvement followed. After intradermic inoculation, characteristic vesicles appeared on the 2nd day with no encephalitic complication. Corneal injection was followed by a keratoconjunctivitis on the 2nd day without cerebral involvement. Finally, intracerebral inoculation of neurovaccine virus was without any specific action. Thus the neurovaccine produced milder lesions in the guinea pig than in the rabbit, and in the guinea pig it was inactive in the brain.

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[16] Flexner, S., *J. Exp. Med.*, 1928, xlvii, 9.

It is evident that the neurovaccine virus, although distinct from herpes virus, acts in rabbits and guinea pigs in a manner comparable to the Levaditi C virus itself. For, while it produces an encephalitis in rabbits, it does not do so in guinea pigs. It does, however, induce local lesions in guinea pigs after intradermal, corneal, and testicular injection, which are manifest, as a rule, 2 days after inoculation.

In combining neurovaccine virus with the Levaditi C strain of herpes virus in the effort to evoke an encephalitogenic effect of the latter in guinea pigs, it was believed that success might follow the injection of the two viruses at different times, so that both would eventually operate at the same period with maximum intensity. We had already determined this period for neurovaccine virus, namely, 2 days. Earlier in this paper, we have shown that the time of maximum activity of the Levaditi strain of herpes virus in the brains of guinea pigs is from 4 to 6 days after its intracerebral injection.

Combined Effects of Levaditi Herpes Virus and Neurovaccine Virus.

The following tests were made in a study of the concomitant effects of the Levaditi virus and neurovaccine virus.

In carrying out the experiments, when virus was to be injected in the brain, the usual dose of 0.15 cc. of a 10 per cent saline suspension of brain containing active virus was used, unless otherwise stated. For corneal and skin injections, the methods of Flexner and Amoss[8] were employed. For intratesticular inoculation of neurovaccine virus, 0.5 cc. of a 10 per cent rabbit brain suspension, containing active virus, was introduced into each of the testes after injury of these organs by repeated needle puncture. In each instance the specific activity of the viruses employed was determined by inoculation into corresponding tissues of susceptible control animals. The period of observation lasted for from 15 days to at least 1 month.

The combinations of viruses injected into guinea pigs, and the results obtained follow.

1. Levaditi C strain of herpes virus in the brain, and 2 days later neurovaccine virus in the cornea. Typical neurovaccine virus keratoconjunctivitis resulted, from which the animals recovered without cerebral involvement.

2. Levaditi herpes virus in the brain, and 2 days later neurovaccine virus in the shaved abdominal skin. There followed no encephalitis, only neurovaccine dermal lesions, from which the animals promptly recovered.

3. Levaditi herpes virus in the brain, and 2 days later neurovaccine virus in the testes. Orchitis developed but no encephalitis.

4. Levaditi herpes virus in the brain, and at the same time neurovaccine virus also in the brain. Since both viruses are inactive in guinea pigs by this method of injection, but produce characteristic encephalitis in rabbits after intracerebral inoculation, guinea pigs were subjected to inoculation simultaneously as follows: (*a*) the usual amount (0.15 cc.) of both viruses was injected separately in each hemisphere, and in another series larger amounts (0.2 cc.) were tested; (*b*) usual quantities (0.15 cc.) of the two viruses mixed *in vitro* were introduced into one hemisphere and in another test larger doses (0.2 cc.) were employed. None of these animals developed encephalitis.

It was not found possible, therefore, to implant the Levaditi virus on the cerebrum of guinea pigs by combining it with neurovaccine virus in such a way as to permit both to act with maximum effects at the same time.

Cross-Immunity in Guinea Pigs. Levaditi and H.F. Strains.

Cross-immunity in rabbits between the Levaditi and the H.F. strains of herpes virus has been determined by Flexner and Amoss.[17] The tests to be reported relate to the corresponding effects in the guinea pig.

Three series of experiments were made at different times, each series containing 3, 6, and 3 guinea pigs immunized with Levaditi herpes virus. In addition, guinea pigs and rabbits were included to serve as controls of the activity not only of the Levaditi virus used in the immunization, but also of the H.F. strain given as a test injection later.

The procedure of immunization consisted in injecting guinea pigs intraperitoneally with 2 cc. of a 25 per cent saline suspension of fresh rabbit brain containing active Levaditi virus. Four to five such inoculations were made at 4 to 7 day intervals. From 7 to 10 days after the last injection, the guinea pigs were given intracranially 0.2 cc. of a 10 to 20 per cent suspension of either guinea pig or rabbit fresh brain containing the H.F. strain of herpes virus. The control animals showed characteristic herpes-virus encephalitis with death in 6 to 7 days, while the immunized guinea pigs remained well.

Cross-immunity has therefore been shown to exist in the guinea pig between the Levaditi virus and the H.F. strain of herpes virus. Moreover, by repeated intraperitoneal injections of the Levaditi virus, during which no apparent signs of disease were noted, a solid immunity was produced.

[17] Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1925, xli, 357.

DISCUSSION AND SUMMARY.

A number of methods have been employed in attempts to induce encephalitis in guinea pigs with the Levaditi C strain of herpes virus. Some of these consisted of different modes of inoculation of the virus itself and others of different ways of combining it with vesicular stomatitis and neurovaccine viruses so as to obtain the concomitant effects of both. In still another test the Levaditi virus was combined with the neurovaccine in a manner calculated to bring about the maximum action of each at the same time. By all these methods, the Levaditi virus failed to evoke the characteristic encephalitis which this specimen is capable of inducing uniformly in rabbits. On the other hand, when the Levaditi herpes virus is inoculated into the brain of guinea pigs in conjunction with suitably timed corneal injections, it acquires active encephalitogenic properties.

The results just noted suggest several considerations:

1. The possibility of increasing the virulence of a filtrable virus by animal passage in a special manner. It is not likely that the increase as observed was due to dosage, for after the virus acquired its encephalitogenic property for guinea pigs, the usual amounts of virus suspensions sufficed to induce, in a uniform way, typical encephalitis.

2. The opinion previously expressed by Flexner[2] that the guinea pig serves merely to separate weak from strong strains of herpes virus is supported: for only according to the particular method described, could the encephalitogenic power of the Levaditi virus be developed and the weak be converted into a strong herpes strain. With the acquisition of this power, the Levaditi virus acted in precisely the same manner as strong herpes strains both in the guinea pig and the rabbit. Moreover, it was shown in guinea pigs that cross-immunity occurs between weak and strong strains.

3. The two samples of neurovaccine virus employed were incapable of inducing encephalitis in guinea pigs after intracutaneous, intratesticular, corneal, or intracerebral inoculation, although they were actively encephalitogenic in rabbits. In spite of the fact that the vaccine virus and herpes virus are different, as shown by the histopathology and absence of cross-immunity, they behave in the same way when injected into the brain of the guinea pig. The failure of the concomi-

tant action of both viruses to induce encephalitis in the guinea pig suggests that the association of two viruses, under the experimental conditions outlined, is incapable of inducing encephalitis, if either, by itself, is non-encephalitogenic.

4. The serum from normal guinea pigs may neutralize a weak (Levaditi C) but not a strong (H.F.) strain of herpes virus; but the neutralizing action of the serum on Levaditi C virus is not uniform.

5. The Levaditi strain of virus can increase in quantity in the brain of the guinea pig to a degree which permits detection and yet fails to evoke any distinctive clinical picture or definite histopathological changes.

6. Repeated intraperitoneal injections of Levaditi virus in guinea pigs elicit no signs of infection, yet they induce a solid immunity to strong strains of herpes virus.

STUDIES ON INDIFFERENT STREPTOCOCCI.

I. Separation of a Serological Group—Type I.

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In the search for the etiological agent of rheumatic fever, attention has frequently been called to the possibility that streptococci may in some way be concerned in the production of the disease. Mostly the suggestions have dealt with organisms of the *viridans* group, less frequently with the hemolytic group, but only recently has it been proposed by Small (1) and Birkhaug (2) that the indifferent streptococci must be considered in this connection. This newer view-point is explainable when it is remembered that the bacteria isolated during life or at necropsy from patients with the disease have usually been *Streptococcus viridans*, and that the demonstration of the indifferent varieties has been a much less common occurrence.

The existence of the indifferent streptococci has been a matter of common knowledge for some decades.

Mandelbaum (3), who mentioned their occurrence in the throat and elsewhere, regarded them as simply a variety of saprophytic organism. Zangemeister (4), commenting upon their lack of virulence, considered them apparently as a degraded form of *S. hæmolyticus*, and believed that under proper conditions certain of them could assume the properties of the latter organism. Their cultural characteristics were dealt with by Brown (5), and a tentative classification upon the basis of certain fermentation reactions was advanced.

That the indifferent streptococci were not completely apathogenic was suggested by the observations of Kinsella (6), who recovered them in four instances from the blood stream of patients suffering from subacute bacterial endocarditis. Furthermore, Rosenow (7) on a few occasions isolated them from joint punctates of acute rheumatic fever patients. Working with a strain grown from the blood of a patient suffering from the latter disease, Small (1) demonstrated that inoculation of this organism intravenously into rabbits resulted in the production of lesions which strikingly resembled those described by Bracht and Wächter (8)

and by Thalhimer and Rothschild (9) and Cecil (10), and others following similar injections of *S. viridans*. Treatment of patients with an antiserum prepared with this strain produced effects recalling those reported two decades ago by Menzer (11) and others as resulting from the treatment of acute rheumatic fever patients with polyvalent antistreptococcus serum. More recently Birkhaug (2), Kaiser (12), and later Swift, Wilson, and Todd (13), have called attention to the fact that certain strains of indifferent streptococci possessing in common the property of fermenting inulin yield culture filtrates which on intradermal injection in moderate dilution produce reactions in individuals suffering from rheumatic fever as well as from other conditions. Perhaps the most striking observation in this connection is the fact that heating this filtrate in boiling water for 1 hour augments its "toxicity" (13). Birkhaug has also suggested that his inulin-fermenting organisms show a certain degree of serological interrelationship.

In order for different workers in a field such as this to be able to compare results satisfactorily, some sort of classification of the organisms in question is desirable. Despite the difficulties encountered by others in classifying streptococci of the hemolytic and *viridans* types, it seemed of interest to examine a number of strains of indifferent streptococci, and to determine whether any simple serological grouping was possible. For this purpose 159 strains were secured. Sixteen of these were obtained through the kindness of Dr. Birkhaug, including the R F 1 strain with which much of his work was carried out. These were all inulin-fermenting organisms of the type described in his paper. Later, as the work was in progress, Dr. Small kindly forwarded two cultures, R 1 and R 9; the former was that strain originally isolated and described by him (1). The remaining 141 were secured from the throats of patients suffering from various diseases, including rheumatic fever, from the throats of normal individuals, or from the interior of tonsils removed at operation. Two of our strains were isolated from blood cultures of patients with acute rheumatic fever.

Methods.

Culture material was obtained from the throats with sterile swabs and from the interior of excised tonsils after sterilization of the surface, and the initial seeding was made upon plain blood agar plates. After an incubation period of 24 to 36 hours colonies of indifferent streptococci were transferred to plates of Birkhaug's medium (2) from which the bile was omitted. These plates served as a rough means for the separation of the inulin-fermenting and non-fermenting types. From these plates strains of both varieties were chosen, and subsequently were

kept in stock culture in blood broth. Final determinations of the capacity of the organisms to ferment inulin and salicin were carried out with tubes of Hiss serum water. Incubation was prolonged for 10 days before negative results were recorded.

Antisera were prepared by the intravenous inoculation into rabbits of whole 24-hour cultures in phosphate broth pH 7.8 containing 0.05 per cent of dextrose. The injections were given semiweekly over a period of 6 to 8 weeks During the first 2 weeks amounts of 0.5 cc. and 1 cc. respectively of heat-killed culture were administered. Thereafter living culture was employed in doses of 0.5 cc., 1.0 cc., and finally of 2.0 cc. The latter dose was repeated in case trial bleedings failed to reveal a satisfactory titer of agglutinin. The animals were exsanguinated 8 or 9 days following the last dose. By this means it was a simple matter to obtain sera which would agglutinate the homologous organisms in dilutions of 1:5000.

For carrying out agglutinations, the bacteria were grown for 24 hours in phosphate broth pH 7.8 containing 0.05 per cent dextrose. Usually the resulting culture formed a homogeneous, turbid suspension, which for the purpose in hand was extremely satisfactory. In a few instances concentration with the centrifuge was necessary to obtain a sufficiently dense suspension; and occasionally growth was so granular as to render the culture useless for agglutination. Antisera were diluted 1:125, 1:250, 1:500, and 1:1000 in phosphate broth; 0.5 cc. of each of these dilutions was mixed in small tubes with 0.5 cc. of bacterial culture; all tests were recorded in terms of the final dilution of serum which resulted. On each test day a series of similar dilutions of normal rabbit serum was employed with each strain under investigation. Suspension controls were set up, in which 0.5 cc. of culture was mixed with 0.5 cc. of phosphate broth. All tubes were kept in an incubator at 56° for 3 hours, at the end of which time readings were recorded.

Antigens for use in the precipitation test were prepared as follows: 35 cc. of a 24-hour growth in 1 per cent dextrose broth were centrifuged, and the sedimented organisms were suspended in 10 cc. of N/100 NaOH in physiological salt solution. In a few instances in which growth was light, as estimated roughly from the volume of the packed organisms, smaller amounts of the alkaline solution were used. These suspensions were permitted to stand overnight in the ice box, at the end of which time they were centrifuged. The clear supernatant fluid was acidified with 10 per cent acetic acid, whereupon a rich precipitate of acid-insoluble protein appeared. This was permitted to flocculate overnight in the ice box, and was then centrifuged off. The water-clear supernatant fluid was neutralized with N/1 NaOH, and was then ready for use. In carrying out the tests 0.1 cc. of antiserum was placed into each of a series of small tubes, and the antigen was added in amounts of 0.2, 0.1, and 0.025 cc.; sufficient normal salt solution was added to make the final volume 0.5 cc. A control series was always employed in which the same amounts of antigen and salt solution were added to 0.1 cc. of normal rabbit serum. The tubes were kept in the water bath at 37°C. for 2 hours, following which they were placed in the ice box; readings were made the following

morning. The resulting precipitates were found to have gathered in the bottom of the tubes in the shape of coherent discs, which strongly resembled those produced when pneumococcus specific soluble substance is mixed with its corresponding antiserum; this suggested the possibility that substances of similar nature were extracted by this simple procedure from the indifferent streptococci.

Parallel agglutination and precipitation tests were carried out as above outlined with each of the 159 strains. At first there were employed four sera, prepared respectively with two of Dr. Birkhaug's strains, and two of our own. Inasmuch as it was soon found that interchangeable results were obtained with all four sera, it was considered permissible to perform all of the agglutination tests with a single serum (R 867), and all of the precipitations with another (R 862). These sera were of approximately the same titer. The first was prepared against Strain Q 88 E, which was isolated from the blood stream of a patient with rheumatic fever. The second was prepared against Strain RF 24 T, secured from Dr. Birkhaug and originally isolated by him from the throat of a patient with the same disease. Results were recorded in the usual way with + signs, ++++ being the maximum; and except for a few instances which will be considered below the results of the two tests were found to check rather closely.

RESULTS.

In Table I are given in summary the results of the observations upon these 159 strains. In the group designated "Inulin fermentation prompt," acid and clot were formed in the Hiss serum water within a period of 48 hours. The group designated "Inulin fermentation delayed" required longer periods of time, and occasionally clot formation remained in abeyance.

As the result of these observations it is possible to separate the strains into two large groups. The first consists of those organisms which show strong agglutination and precipitation reactions with the sera employed. This serological uniformity seems sufficient warrant for the inclusion of all these strains within a single category, which may be designated as Type I. As might be expected, the organisms are not absolutely identical, as revealed by minor variations in the strength of the reactions. Included under Type I are a few strains which fail to conform exactly to the above conditions: (1) some which show spontaneous agglutination, but which can be typed by means of the precipitin reaction, (2) others which agglutinate to titer in the Type I serum, but from which it is not possible to extract the precipitating antigen in adequate concentration by the standard method. In the

latter case it may be tentatively assumed either that in a few strains the antigen is present within the bacterial cell in materially smaller amounts than usual, or that, inasmuch as the antigens from different strains do not of necessity possess the same degree of stability, an

TABLE I.

	Inulin fermentation	Agglutination Serum R 867	Precipitation Serum R 862	Strains
Type I	Prompt	++++	++++	45
	"	++++	+++	11
	"	+++	++++	5
	"	+++	++ to +++	5
	"	Spontaneous	+++ to ++++	5
	"	++++	+ to ++	9
	"	+++	+	1
	"	++++	− to ±	2
	"	+++	− to ±	3
Total				86
Group X	Prompt	++	− to ±	2
	"	±	++	1
	"	± to +	± to +	7
	"	− to ±	− to ±	3
	"	Spontaneous	+ to ++	4
	"	"	− to ±	15
	Delayed	++	+	1
	"	+	+	2
	"	+	±	1
	"	−	−	3
	"	Spontaneous	+	1
	"	"	− to ±	8
	Negative	+	± or −	5
	"	−	± or −	14
	"	Spontaneous	± or −	6
Total				73

occasional one may be injured by the reagents employed. All the strains of Type I ferment both inulin and salicin promptly.

The second group comprises those organisms which agglutinate weakly or not at all in Type I antiserum at a dilution of 1:250 or higher, and which fail to yield strongly precipitating extracts by the

TABLE II.

Class	Strain	Inulin	Salicin	Agglutination: Serum R 167 Serum dilutions				Precipitation: Serum R 862 Antigen in cc.		
				1:250	1:500	1:1000	1:2000	0.2	0.1	0.025
Type I	Q 88 E	+ prompt	+	++++	++++	+++	++	++±	++++	++
	RF 24 T	+ "	+	+++	+++	++	+	++±	+++	++
	Q 166 B	+ "	+	++++	++++	+++	+	+++	+++	+±
	P 5 B	+ "	+	++++	++++	+++±	+	±	(±)	−
	P 86 B	+ "	+	Spontaneous				++++	++++	++±
Group X	RF 156	+ prompt	+	+	+	−	−	±	(±)	−
	R 9	+ "	+	++	+	−	−	+	±	−
	P 28 B	+ (4 days)	+	Spontaneous				?	(±)	−
	P 71 B	−	+	−	−	−	−	±	(±)	−
	P 128	−	+	Spontaneous				±	(±)	−
	R 1	−	+	−	−	−	−	?	−	−
	P 16 B	−	−	−	−	−	−	−	−	−

standard method employed. In general the same correspondence appears between the results of the agglutination and precipitation tests. It is recognized that in still lower dilutions of the serum agglutination might occur, and in a few instances this possibility has been tested and found to be a fact, but such slight cross reactions in a high titer serum are not of significance from the point of view of the present study. A greater percentage of the strains in this group show a tendency to flocculate spontaneously than is the case with those of Type I. The differentiation of the strains of this group from those of Type I is distinct, but the data are insufficient to warrant the delimitation of further types at present. These strains may, however, be placed together provisionally as Group X, with the understanding that this is a frankly heterogeneous collection from which in the future it may well prove possible to separate further definite types. The members of this group may vary in their capacity to ferment inulin: in some it is well marked; in others it becomes apparent only after a lapse of several days; while in the case of many strains it is entirely lacking. The group has been further studied from the point of view of salicin fermentation. Slight precipitation occasionally occurred with the Type I serum when extracts from salicin-fermenting organisms were employed, but was never observed in the case of non-salicin fermenters. In view of the fact that all of Type I strains are salicin fermenters this cross relationship is noteworthy and suggests that the strains which do not ferment salicin are the least closely related to Type I of any studied.

In Table II is presented a composite protocol, which serves to illustrate the manner in which the data were collected and the grouping of the individual strains arrived at. It is possible that certain of the weak precipitin reactions recorded in Group X were traceable to small protein residues in the bacterial extracts. Inasmuch as only the acid-insoluble protein was removed, the solutions were obviously far from containing one antigen only.

It should be noted that in our hands Strain R 1 has consistently failed to ferment inulin unless adapted following six to eight transfers in inulin serum water, or unless faulty inulin was employed. In Small's original report this strain is designated as an inulin fermenter. This divergence may be explainable by differences in the media used.

Unless great care is observed in sterilization the inulin will be fermented by many ordinary non-fermenters. Certain lots of inulin are also easily fermented by most indifferent streptococci. It is, therefore, necessary to control carefully each new lot of media. In this work if a strain failed to ferment salicin but fermented inulin the inulin was always found to be at fault.

DISCUSSION.

The existence of a group of indifferent streptococci sufficiently compact to warrant separation as a distinct type suggests certain analogies to conditions within the pneumococcus family. As far as the present observations indicate, the antigen complex of these streptococci contains a substance which by the analogy of disc precipitation in immune serum may be regarded as comparable to the pneumococcus specific substance. This analogy is strengthened by the fact that the active substance is destroyed by boiling for 30 minutes with N/10 HCl, and that as this hydrolysis proceeds copper-reducing substances appear in the solution. The usually close agreement between the results of the agglutination and precipitation reactions suggests that it is this same substance which is responsible for both of the immune reactions, and that the compactness of Type I results from the presence within a great number of strains of indifferent streptococci of soluble substances identical or very similar chemically. Such a concept is comparable to that developed by Avery and Heidelberger (14) in the case of the pneumococci, and by Lancefield (15) for certain strains of *Streptococcus viridans*, but is at variance with conditions as they exist within the family of the hemolytic streptococci (16, 17). Efforts to identify the soluble substances of Type I with the species-specific soluble substance of the hemolytic streptococci have been unsuccessful. On the other hand, a few strains of *Streptococcus viridans* have been encountered which have shown agglutination in Type I serum, and from these strains it has been possible to prepare extracts which precipitate such serum with the formation of fairly compact discs. Such strains of *Streptococcus viridans*, therefore, contain antigens in common with Type I indifferent streptococci. Similar instances of cross agglutination have been reported by Birkhaug. Whether such observations have more than an academic interest is at present a matter of conjecture.

SUMMARY AND CONCLUSIONS.

Serological study of a large number of strains of indifferent streptococci has revealed the existence of a large homogeneous group to which the designation Type I has been applied. It is recognized that members of Type I are not necessarily identical, and that further division into subtypes may be feasible. All strains of Type I ferment inulin and salicin.

The remaining strains are referred to as belonging to Group X. They are distinguished only by their failure to react strongly with Type I serum. While at present this group must be regarded as quite heterogeneous, further work may reveal the presence of other as yet undefined types now included within its limits. The organisms of this group vary in their fermentative reactions with both inulin and salicin.

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STUDIES ON INDIFFERENT STREPTOCOCCI.

II. Observations on the Distribution of Indifferent Streptococci in the Throats of Rheumatic and Non-Rheumatic Individuals.

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Within the past year the attention of investigators in the field of rheumatic fever has been sharply directed to the indifferent streptococci by the work of Small (1) and of Birkhaug (2). The former has cultivated organisms of this type from patients with the disease, and has prepared a therapeutic serum with which results are reported similar to those obtained with other varieties of antistreptococcus sera (3). He has assumed that these bacteria are to be regarded as the specific causative agents of the disease. Birkhaug has recovered streptococci of the same type from blood, throats, tonsils, and feces of patients suffering from the disease, and with culture filtrates of these organisms has obtained a high percentage of positive skin reactions in individuals "stigmatized by rheumatic fever or its syndromes." Neither of these investigators has adduced convincing evidence that indifferent streptococci are to be more seriously considered in connection with this disease than are streptococci of the *viridans* type.

It might be thought that, if the indifferent streptococci played a special rôle in the production of rheumatic fever, they should be particularly abundant in the throats of patients during the course of the disease or during early convalescence. If such were the case, they should be found with especial ease during the period of acute tonsillitis or pharyngitis which usually constitutes the initial stage of the illness and is frequently observable at the time of hospitalization. It seemed, therefore, of interest to study the flora of the throats of a group of patients suffering from one or another of the acute manifestations of rheumatic fever. As control groups there were studied (1)

patients who had recovered from the disease, symptom-free for at least several months and usually for a year or more; (2) patients suffering from other conditions; (3) normal individuals. The persons constituting the last two groups were free from any condition suggesting the rheumatic syndrome, and with one or two exceptions had never suffered from any of its manifestations.

Methods.

Cultures were obtained from throats by simple swabbing of the tonsils, or tonsillar fossæ, and pharynx. Initial seedings were made upon blood agar plates, spread radially with a platinum loop. At first plates of the inulin-bile-serum agar medium described by Birkhaug (2) were also employed, but these were found to present no unusual advantages and were soon abandoned for purposes of isolation. After an incubation period of from 24 to 36 hours the plates were studied, and a rough estimate was made of the percentage of indifferent colonies present. From six to eight colonies were then picked to blood agar plates, and after appropriate incubation smears were examined microscopically. All strains of indifferent streptococci were then transferred to plates of 1 per cent inulin agar enriched with chest fluid, and at the end of 48 hours the relative numbers of inulin-fermenting and non-fermenting strains were recorded. This method served roughly for initial differentiation, although with many strains it was found that inulin fermentation proceeded readily in tubes of Hiss serum water though it had failed to become evident when the plate method was employed. From the inulin agar plates strains were transferred to tubes of blood broth which were preserved in the ice box for further study. Usually two strains from each culture were thus preserved.

Final determinations of the capacity of the organisms to ferment inulin and salicin were made with tubes of Hiss serum water containing the appropriate carbohydrate. Inocula consisted of 0.2 cc. of a 24-hour culture in broth containing 0.05 per cent of dextrose. No result was recorded as negative until a 10-day period of incubation had elapsed. Two strains from each culture were examined serologically according to methods described previously (4). In most instances both agglutination and precipitation reactions were employed; after the close correspondence between the results of these two methods had been established, it was considered permissible in the case of a few strains to employ the reaction of agglutination alone.

RESULTS.

In Table I are presented data based upon the total number of cultures studied, analyzed with reference to the occurrence of indifferent streptococci.

Regardless of the presence or absence of rheumatic fever, there seems to be a tendency for these organisms to be found somewhat more frequently in the throats of individuals suffering from some condition of

TABLE I.

Incidence of Indifferent Streptococci in Throat Cultures.

	Total cultures	Occurrence of indifferent streptococci	
		Present	Absent
Active R.F. or early convalescence	34	29 (85%)	5 (15%)
R.F. recovered	26	24 (92%)	2 (8%)
Other conditions	36	32 (89%)	4 (11%)
Normal individuals	49	37 (76%)	12 (24%)
	145	122 (84%)	23 (16%)

TABLE II.

Incidence of Indifferent Streptococci in Throats of Individuals.

	Total persons	Occurrence of indifferent streptococci	
		Present	Absent
Active R.F. or early convalescence	28	26 (94%)	2 (6%)
R.F. recovered	23	21 (91%)	2 (9%)
Other conditions	36	32 (89%)	4 (11%)
Normal individuals	45	36 (80%)	9 (20%)
	132	115 (87%)	17 (13%)

disease than in those of normal persons. The differences are not, however, very striking. It is noteworthy that the bacteria under consideration were harbored quite as frequently by patients free from any taint of rheumatism as by those who were definitely suffering from

some manifestation of the disease. Certainly it would appear justifiable to conclude that the indifferent streptococci are widely distributed, carried more or less constantly by roughly 85 per cent of the population, and that there is a slight and entirely non-specific tendency for them to be found more frequently in the throats of hospital patients than in those of normal individuals. In view of the innate inexactness of the method, the results in the various groups are surprisingly concordant. Inasmuch as in a few cases more than one culture was made from a single individual, and as when indifferent streptococci are scarce they may be missed in the first culture and be found in the second, or *vice versa*, it has seemed desirable to present

TABLE III.

Percentage Incidence of Indifferent Streptococci in Throat Cultures.

	Total cultures	1–5%*	6–15%	16–30%	Above 30%	Present**
Active R.F. or early convalescence...	34	17 (50%)	5 (15%)	1 (3%)		6 (18%)
R.F. recovered.....	26	16 (62%)	3 (11%)	3 (11%)	1 (4%)	1 (4%)
Other conditions.....	36	22 (61%)	4 (11%)	3 (8%)	2 (6%)	1 (3%)
Normal individuals.....	49	22 (45%)	9 (18%)	5 (10%)		1 (2%)

*Includes a few early cultures described as containing "few" or "very few."
**Includes a few of the first cultures in which estimates were not made.

Table II, in which the data are analyzed in terms of individuals rather than of total cultures.

Comparison with Table I shows that there is a tendency for the percentage of negative results to decrease when repeated cultures are taken from the same group of individuals. In all probability every person now recorded as free from these streptococci would eventually be found to harbor them, though in very small number, if the cultural procedure were repeated with sufficient frequency.

Although the indifferent streptococci were found no more frequently in the throats of one group of individuals than in those of another, it might be considered that in the case of patients suffering from some active manifestation of rheumatic fever these bacteria would consti-

tute a much greater percentage of the total flora than would be the case with other individuals. In Table III are presented data which bear upon this point.

The cultures from each group of individuals have been divided into subgroups, according as the indifferent streptococci constituted 1–5 per cent, 6–15 per cent, 16–30 per cent, or more than 30 per cent of the total flora, and the total number of cultures in each subgroup have been tabulated, together with the percentage of the main group formed by each subgroup. That the percentages under each group do not add up to 100 per cent results from the omission of a column for the negative cultures.

TABLE IV.

Incidence of Groups of Indifferent Streptococci in Throats of Individuals.

	Total strains	Distribution into groups	
		Type I	Group X
Active R.F. or early convalescence	43	21 (49%)	22 (51%)
R.F. recovered	32	13 (40%)	19 (60%)
Other conditions	43	20 (46%)	23 (54%)
Normal individuals	56	30 (53%)	26 (47%)

In slightly more than half of the individuals indifferent streptococci were found to constitute 1–5 per cent of the total throat flora, irrespective of the presence or absence of rheumatic fever or its stigmata. Thus, in 45 per cent of all the cultures from normal individuals these organisms were estimated as forming 1–5 per cent of the total flora. Reference to the remainder of the table shows that there was no tendency for these streptococci to occur in greater abundance in the throats of patients with rheumatic fever than those of other patients. In fact, some of the highest percentages recorded were found in persons free from any taint of rheumatism, while, conversely, it was several times observed that during very acute relapses of this disease there was a tendency for the plates to be crowded with *Streptococcus viridans*, largely to the exclusion of other types of organism.

In Table IV the total number of strains studied from each group of individuals is analyzed with respect to their serological characteristics (4).

In view of the fact that only one or two strains from each positive culture were studied, so that the element of chance played a large part, there is a remarkable agreement between the percentages for the various groups of persons. The probability that an indifferent streptococcus chosen at random from a throat culture will belong to Type I or will fall into the heterogeneous Group X is apparently independent of the condition of health of the individual from whom the culture is taken. In other words, there is no greater tendency for the organisms of Type I to be found more frequently in the throats of one group of individuals than in those of another, and in rheumatic fever there is no tendency for the incidence of this type to vary in either direction.

DISCUSSION.

The isolation of organisms of various types from lesions of rheumatic fever has been a more or less frequent occurrence. Many of the reports are open to grave suspicion of error in technique or of interpretation. The best accredited results are those in which streptococci of various types have been found. Never, however, has it been possible to secure experimental evidence that these organisms are directly concerned in the production of the disease. Their injection into laboratory animals has never been followed by the development of lesions closely resembling those of rheumatism. The pathological findings in the joints have been usually those of a proliferative and destructive arthritis (5, 6) while in the heart vegetative endocarditis has been present, together with the Bracht-Wächter bodies (7) in the myocardium. This has been the case regardless of whether the streptococci were of the indifferent or *viridans* type. Recent investigations (8, 9) have suggested that the failure of all these attempts may not mean that the bacterium used was unassociated with rheumatic fever, but that the error has been one of method. That is, the preliminary induction of the necessary tissue allergy in the experimental animal may be the *sine qua non* which hitherto has been neglected. Given the proper condition of tissue sensitization any streptococcus possessed

of the requisite allergizing capacity may be able to give rise to the peculiar condition called rheumatism.

The most recent organism to be ascribed a specific etiological rôle in rheumatic fever has been the indifferent streptococcus. The first object of this present investigation was to determine the extent of the distribution of these organisms among selected samples of the population and the percentage of the total flora of the throat which they formed. In spite of the fact that bacteriologists have been familiar with these organisms for decades, no such study has to our knowledge ever been carried out. With these fundamental facts established, it was then proposed to compare the flora of the throats of a number of groups of individuals, to determine whether in the case of patients suffering from rheumatic fever they occurred in greater incidence or in higher percentage. Such was not the case. There was found a slightly higher incidence of indifferent streptococci in the throats of hospital patients than in the throats of normal persons; but from the present study it is apparent that these bacteria are no more widely distributed among patients suffering from rheumatic fever than among other groups of patients. In the majority of throats they are present in approximately the same percentage relative to the total flora. Brown (10) has observed that in inflammatory conditions of the throat they tend to be overgrown by other organisms, notably the hemolytic streptococcus; and in a few cases of acute rheumatic fever included in the above tables there has been a tendency for *Streptococcus viridans* to displace them in similar fashion. Certainly such findings are not in harmony with the claim that these organisms are the sole cause of the disease.

Future investigation will probably determine the rôle which the indifferent streptococci actually play in the disease. Doubtless they must be considered along with the other types of streptococci in any comprehensive theory of its etiology. That they are not simple saprophytes is suggested by the facts that occasionally they may be the cause of bacterial endocarditis (11), that they are often efficient allergizing agents (12), and that they possess the capacity of yielding "toxic" filtrates (2, 13, 14). Therein they do not differ from many strains of *Streptococcus viridans*, and consequently are no more entitled to special consideration than are the latter.

It may be objected that the cultural and serological types here reported are not exclusively those used by Small (1) in the preparation of his SCA antiserum, and hence the validity of these conclusions may be questioned. In this connection attention should be directed to the fact that among the strains investigated in these two studies there were RF 1 recovered by Birkhaug from blood cultures of a rheumatic fever patient and Q 88 E and Q 97 EA similarly recovered by us from patients with the active disease. All of these fell into Type I. The R 1 strain and R 9 strain recovered by Small differ from each other and are also heterogeneous to Type I. While these strains are closely related as judged from their effect on blood agar, still their serological heterogeneity casts the same doubt on their specific etiologic rôle as was cast on *Streptococcus viridans* and for the same reason. We feel, therefore, that indifferent streptococci play a rôle in the production of this disease similar to that exerted by the green-forming streptococci.

SUMMARY AND CONCLUSIONS.

Indifferent streptococci occur in comparatively the same abundance in the throats of patients suffering from rheumatic fever or early in convalescence from the disease as they do in those who have recovered from the disease, or in those of patients suffering from other diseases.

There is a slightly increased incidence of these microorganisms in the throats of hospital patients as compared with those of normal individuals.

Type I occurs with comparatively equal frequency and abundance in the throats of all four classes of individuals studied.

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FURTHER EXPERIMENTS WITH THE INTRADERMAL PNEUMOCOCCUS INFECTION IN RABBITS.

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In a previous communication (1) we have described the characteristic symptom-complex brought about by infecting rabbits intradermally with Type I pneumococci. The following paper is a continuation of that report.

I.

Specific Serum Therapy.

Since we believe that the rabbit experiment as described in our first paper promises a method of determining the therapeutic potency of specific antipneumococcic serum more satisfactorily than the now prevalent mouse protection technic we have given considerable attention to the effects of serum administration on the course of the pneumococcus infection of rabbits, both in regard to the local and general phenomena.

The following points have already been established:

1. That animals recover promptly after the intravenous administration of large amounts of antipneumococcic serum.

2. That there are four important features of this induced recovery, as follow: (*a*) the immediate and permanent disappearance of organisms from the circulating blood; (*b*) a drop in temperature to normal levels within 24 hours; (*c*) the disappearance of organisms from the local lesion in the course of 4 to 20 hours; (*d*) loss of inflammatory color of the local lesion within 24 hours, usually accompanied by epidermal desquamation.

3. That for a given serum at a given stage of the disease there may be established a *minimal effective dosage* (M.E.D.) such that larger

amounts give no added therapeutic value and smaller amounts are insufficient to bring about recovery.

Minimal Effective Dosage at Various Stages of the Disease.—It is a common clinical observation that the amount of serum which might be used effectively on the 1st or 2nd day of lobar pneumonia is insufficient if given at a later time. Some clinicians have even stated that after the infection has progressed beyond a certain point no amount of serum will save.

The question of a quantitative element has been studied in the experimental "dermal pneumonia" of the rabbit by determining the minimal effective dose of a given serum for each of three stages of the disease. It is necessary to stress again the sharpness of the end-point in determinations of the effectiveness of therapeutic sera. The results of such an experiment are shown in Fig. 1.

This experiment shows that the amount of serum necessary for effective therapy increases very rapidly as the disease progresses and emphasizes the importance of early treatment. Even the time occupied in the ordinary typing process would greatly lessen or entirely eliminate the chances of successful therapy.

For this particular serum, if one assumed a definite relationship between therapeutic dose and body weight of the animal and transferred these proportions to man, the results would be as follow: In the standard rabbit (1500 gm.) we have found it necessary to use serum to the amount of 0.23 per cent of body weight at 24 hours, 0.47 per cent at 48 hours, and approximately 0.67 per cent at 72 hours. This would indicate that in a man of 70 kilos it would be necessary to use 150 cc. of this serum at 24 hours, 325 cc. at 48 hours, and approximately 475 cc. at 72 hours.

In the experimental rabbit condition it is not essential that the minimal effective level be exceeded at a single injection but if multiple injections are used these must not be too far apart and the total quantity must exceed the minimal effective amount for that period. Unless this effective level is exceeded at a given time absolutely no beneficial result has been obtained.

Bloomfield (2) advances the opinion that in lobar pneumonia the usefulness of serum depends upon the presence and seriousness of the bacteriemia. The rabbits which we have studied have shown some

variation in the severity of the bacteriemia at the time of treatment but the minimal effective dosage of the serum appears to be a fixed quantity for a given period and this does not vary with the severity of the bacteriemia. If such a correlation did exist it might be anticipated that ten times as much serum would be necessary at 48 hours

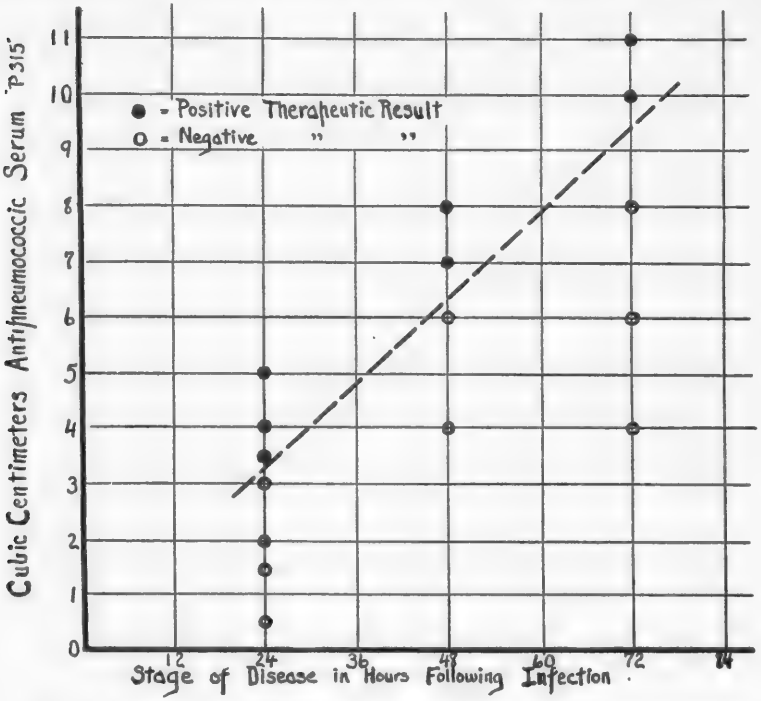

FIG. 1. Determinations of the minimal effective dosage of Antipneumococcic Serum P 315 at various stages of the disease. The diagonal line represents the suggested effective level of therapy.

as was necessary at 24 hours, for the number of bacteria per cc. of blood has often increased by a multiple of 10 or even of 100.

The contour of the suggested boundary curve, shown by the broken line in Fig. 1, suggests that there is a progressive rise in some element which unites quantitatively with the antiserum or quantitatively

antagonizes it. Proponents of the pneumonia toxin theory have advanced the idea that there may be a progressive accumulation of some toxic element in the tissues.

Decision as to the significance of the findings presented in Fig. 1 must await the solution of this question. Our results have shown, however, that there is an experimental basis for early and vigorous treatment of lobar pneumonia, and would seem to point to the immediate use of some sort of polyvalent serum, since the time required for typing greatly decreases chances for success with practicable amounts of serum.

The use of agglutinin titer or protective value of a specifically treated patient's serum in guiding further specific therapy has been suggested by Sutliff (3). The qualitative detection of agglutinins and protective substances in the treated rabbit indicates only that it has been treated; even if such tests are made quantitatively the information gives only the approximate dilution that the therapeutic serum has undergone with the rabbit's own serum. It is obvious that qualitative tests have little value since various antibody levels are necessary at various stages of the disease in order to bring about successful therapy.

The Comparison and Standardization of Antipneumococcic Sera on the Basis of Therapeutic Value.—The earlier workers used agglutinin titer as a basis for comparison of probable therapeutic efficacy of sera. This method has given way to a titration in which mouse protection is the indicator of protective value. The Hygienic Laboratory has established a certain standard and furnishes for comparative purposes a serum which fulfills this requirement. This standard is not based on any known quantitative therapeutic value for cases of lobar pneumonia.

Having found the minimal effective dosage at a given hour in a 1500 gm. rabbit to be a characteristic of the serum and of a constant value for that serum, experiments were planned by which this might be made the basis for the comparison of sera.

Seven sera were selected for comparison. The general plan of work consisted in determining for each of the sera the mouse-protective value, the agglutinin titer, and the therapeutic minimal effective dosage for rabbits at 24 hours.

In determining the mouse-protective value it was assumed that the so called virulence titer of a culture was in reality a measure of the number of minimal fatal doses of virulent pneumococci per cc. for mice. The culture used had a virulence such that 0.000,000,1 cc. would cause the death of a mouse within 4 days. With a definite dilution of this culture and varying quantities of the serum to be tested it was possible to arrive at a numerical value for the protective titer of each serum—this protective value being defined as the number of mouse-protective units per cc. of undiluted serum. The least amount of serum which will protect against a given dosage of pneumococci is determined. By dividing this amount by the number of M.L.D. of pneumococci used, a figure is obtained which represents a unit of serum. The number of such units per cc. of serum constitutes the value which we use for comparison. In every instance except one, 0.1 cc. of culture (100,000 M.L.D.) was employed against varying dilutions of serum. The mouse-protective value which represents the minimal Hygienic Laboratory standard is 5,000,000, according to this system of calculation, but it will be seen that the serum furnished by them for comparison shows a much higher figure.

The agglutinin titer was found by the ordinary technic, with heat-killed pneumococcus suspension as the agglutinogen.

In determining the therapeutic value of each serum, rabbits were treated intravenously at 24 hours following infective intradermal inoculation. Careful studies such as detailed in the previous paper were made before and after treatment. In order to determine the approximate therapeutic zone a preliminary experiment was carried out with widely varying amounts of serum. Then a second experiment was conducted in which the amounts were less widely separated. The rabbit therapeutic value is expressed in minimal effective doses per cc. of serum.

The following sera were used for comparison.

1. Antipneumococcic serum (horse). Hygienic Laboratory Control P 7.

2. Antipneumococcic serum (horse). Massachusetts Antitoxin and Vaccine Laboratory, Lot P 315. This serum is of the type with which physicians are supplied.

3. Antipneumococcic serum (horse). Massachusetts Antitoxin and Vaccine Laboratory, Lot 400–31. An experimental serum prepared by the Zinsser-Grinnell blood clot method (4).

4. Antipneumococcic serum (horse). Lot 440–3–12. An experimental serum: a bleeding at 6 days following a single injection of killed pneumococci.

5. Concentrated pneumococcus antibodies (Felton). Type I, Lot 56. Supplied through the courtesy of Dr. L. Felton.

6. Serum from a rabbit which recovered without treatment. Bled 30 days after the infective inoculation (25 days after critical recovery). Lot 296.

7. Serum of a rabbit immunized by repeated injections of washed heat-killed pneumococci. Lot 301.

The results of the necessarily large number of experiments are condensed in Table I.

The great interest in this table lies in the fact that the mouse-protective doses listed in the column of mouse-protective values show the extraordinary potency of the serum in protecting a highly susceptible animal when serum and organisms are mixed before injection, an experimental condition which is comparable in absolutely no respect to the condition under which the serum is therapeutically used. In the second column under "Rabbit therapeutic value," also calculated for 1 cc., is shown the much diminished potency, though still considerable, when the serum is used in another highly susceptible animal under conditions which, we believe we are justified in assuming, simulate

TABLE I.

Comparison of Agglutinin Titer, Mouse-Protective Value, and Rabbit Therapeutic Value of Seven Sera.

Serum	Mouse-protective value (in terms of fatal doses protected against by 1 cc. of serum, if serum and organisms are mixed *in vitro* before injection)	Rabbit therapeutic value		Agglutinin titer
		Minimal effective dose of serum (amount necessary to bring about recovery if administered 24 hrs. after infection)	Minimal effective doses per cc. of serum	
		cc.		
1	500,000,000	3.0	0.33	80
2	50,000,000	3.5	0.29	40
3	50,000,000	3.5	0.29	40
4	500,000	>10.0	Less than 0.1	0
5	1,428,000,000	0.2	5.0	640
6	5,000,000	3.5	0.29	10
7	50,000,000	0.5	2.0	320

the condition in man, under which serum is therapeutically employed, as closely as this can be done in animal experiments.

It is not altogether surprising that there is not a complete parallelism between figures in the three columns but it is somewhat difficult to account for this in every case. The first point that may well be considered is the therapeutic value of homologous as against heterologous immune sera. Nos. 6 and 7 are from rabbits, and it is to be observed that these sera have a low mouse-protective value in comparison with their relatively high therapeutic value for rabbits. Thus, Serum 7 has only one-tenth the protective value of Serum 1, but its

therapeutic value is six times greater. Compared to Nos. 2 and 3, which have the same protective value, this serum has six or seven times the therapeutic value. The same comparisons hold good for Serum 6. It is obvious that an homologous immune serum, although low in protective value for an heterologous animal, does possess marked therapeutic value as contrasted to the immune serum of a third species. The cells of the treated animal are intimately concerned with any change in the status of the disease and the homologous serum probably owes its advantageous effect to the fact that such proteins are more readily absorbed by the cells.

A consideration of the therapeutic value of the four more potent antisera from horses (Nos. 1, 2, 3, and 5) shows that there is a more definite correlation with the agglutinin titer than with the mouse-protective value. There is a very striking dissimilarity in relative mouse-protective and rabbit therapeutic values.

It is suggested that a method of standardization of antipneumococcic serum, based on the rabbit therapeutic usage as outlined, might prove valuable as compared with the present system. Such a procedure would be no more difficult and only slightly more expensive than the mouse method and the results are quite as regular and definite. It would have the great advantage of measuring that property for which the serum is utilized. Our chief hesitancy in proposing such a method is that we do not know, nor is there any present method of determining, whether the therapeutic value for rabbits does represent a definite measure of therapeutic value in human lobar pneumonia.

II.

Non-Specific Therapy.

In many of these therapeutic experiments large amounts of foreign protein have been injected and it becomes important to separate the specific and non-specific effects. To this end the latter type of therapy has been studied in sixteen cases with normal horse and rabbit sera and typhoid vaccine as the non-specific agents. Various quantities were employed and the cases were usually treated at 24 hours following infection. Four of this series of sixteen survived but this survival rate does not sufficiently exceed that of untreated cases to be considered significant.

Following the intravenous injection, at 24 hours after infection, of 3 to 5 cc. of normal horse serum the temperature may be slightly elevated for a few hours. The number of white cells, already rather low, is not appreciably altered. The most striking result that has been

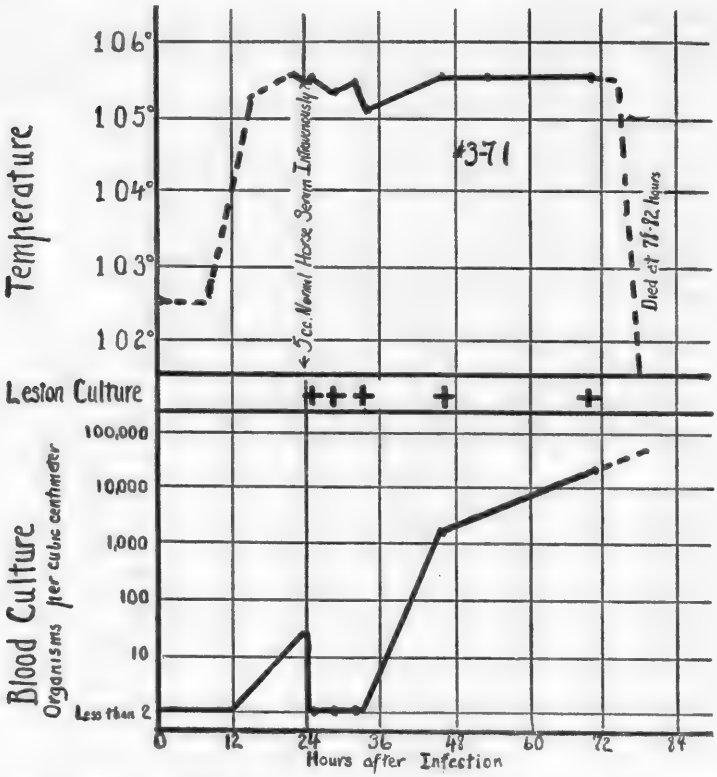

FIG. 2. Chart of findings on Rabbit 3–71 showing the effects of the injection of a non-specific agent.

observed is the immediate disappearance of pneumococci from the circulating blood. The blood may remain free of organisms for several hours but the bacteria then reappear in increasing numbers. A chart of such a case is shown in Fig. 2.

The normal horse serum used in these experiments had no protective action against pneumococci in mice and the result of the treatment of these rabbits, in so far as can be determined, must be due entirely to non-specific factors. This conclusion is supported by the finding that the same result is obtained if typhoid vaccine is administered intravenously.

It has been suggested that the immediate freeing of the blood stream of organisms in the specifically treated case may be due entirely to non-specific factors, but this is probably not the case. That the specific element alone is capable of bringing about this effect is shown in instances of successful intravenous therapy with the homologous (rabbit) immune serum as contrasted to entirely negative results with the homologous normal serum. The injection of normal rabbit serum appears to have no effect upon the number of circulating organisms.

These results show that the non-specific element involved in specific intravenous therapy with "raw" serum must be considered as a definite factor but negligible as compared to the specific element and probably having no bearing on the outcome of any case.

III.

Active Immunity.

In our previous communication (1) the relation of crisis to the development of active immunity in the experimental rabbit disease was discussed. In the following paragraphs we propose to present observations concerning active immunity in convalescent animals and in those previously vaccinated, and to discuss the problem of active immunization in pneumonia.

The Determination of Active Immunity.—Our experience leads us to believe that for the present the only reliable method of determining active immunity is by observing the response to reinfection. Preliminary experiments showed that convalescent rabbits, though immune, still develop a limited localized lesion at the site of intradermal inoculation of undiluted virulent pneumococcus broth culture, and for this reason, before an arbitrary reinfection dose could be selected, it was necessary to study the results obtained with varying amounts of culture.

The method of carrying out this experiment differed from that used in determining the susceptibility of the normal rabbit to varying intradermal dosage of pneumococci, for in the convalescent case the lesion obtained does not exhibit the tendency to spread that is seen in the normal animal. A number of tests may therefore be made on a single animal at the same time. The results of such an experiment are shown in Table II.

On the basis of this experiment it seemed that there would be no particular advantage attached to the use of any one dose for reinfec-

TABLE II.

Susceptibility of the "Immune" Rabbit to Varying Dosage of Pneumococci.

Rabbit 4–28; 22 days convalescent. Entire abdominal area shaved, and varying amounts of culture, diluted to 0.2 cc. in broth, injected intradermally in various separated areas.

Area	Amount of broth culture	Reading at 24 hrs.
	cc.	
1	0.2	Local lesion with definite color and edema; no tendency to spread
2	0.04	As above
3	0.01	Local area of heightened color; no swelling
4	0.002	Trace of color at point of inoculation; no swelling
5	0.001	Very slightest trace of color at point of inoculation; no swelling

With amounts below those given, no sign of inflammation was observed

tion except that with the larger amounts the local lesion would amply safeguard the fact that virulent organisms had been injected. We have therefore used 0.2 cc. of undiluted 18-hour broth culture as a routine. This is injected, as in cases described in our previous paper, well up on the animal's side. Observations as to the character of the lesion and the animal's temperature are made at 24 hours. Blood cultures are not done unless the lesion is widespread, for in our experience the local type of lesion is associated with entirely negative blood cultures.

The resistance to infection has been studied by this method in over 50 cases. The results show some variation but each case falls dis-

tinctly into one of the divisions of a classification that has been devised and is shown in Table III. This classification is based on the type of immune reaction previously described for untreated convalescent cases and upon the assumption that the normal rabbit has no immunity against the pneumococcus.

The first class shown in Table III represents the normal rabbit, which has no immunity against intradermal pneumococcic infection and which after infection undergoes the type of disease that we have described in detail in the previous paper.

Classes 2 and 3 represent those cases which are spoken of as possessing partial immunity. The lesions in these cases are practically

TABLE III.

Classification of Results in Observations on Active Immunity.

Class	Designation of immunity	Readings at 24 hrs.		Course
		Area of lesion	Temperature	
1	−	Widespread	High	Typical febrile 5- to 7-day course
2	+	Widespread	Moderate (less than 105.0°)	Typical course except less severe
3	++	Widespread	Low (less than 104.0°)	Short and mild course
4	+++	Local	Little or no elevation of temperature	Lesion begins to subside in 36 to 48 hrs.
5	+++ +	No lesion or sign of lesion	No change in temperature	

indistinguishable from those in Class 1, but there is a significant lack of severity in the temperature reaction and other objective symptoms.

Class 4 has already been described. It represents the ordinary maximal immunity observed following an attack of the disease. It therefore probably is comparable to *absolute natural immunity.*

Into the fifth class fall those animals which have been immunized by a course of injections of dead pneumococci; that have built up a high antibody content and may be conveniently referred to as *hyperimmune.*

Active Immunity Following Recovery without Treatment.—Since only a small proportion of untreated cases survive an attack of the experi-

mental disease, it has not been possible to study some features of the acquired immunity in the greatest detail. It has been determined that a definite (+++) immunity is acquired and that this immunity persists at a high level for several months. In one case it persisted for 9 months but in two other instances the immunity was not maintained for this length of time. In one case in a series of ten, partial immunity only was observed at the end of the 2nd month. We hope to determine the limits of efficiency of this acquired immunity in future experiments. For the present no absolute conclusion is justified except that the immunity acquired seems to persist for several months but is not permanent.

Active Immunity in Treated Cases.—In repeated instances we have determined that if a rabbit subjected to dermal inoculation is treated within 24 hours with an effective dose of heterologous serum it promptly recovers but after the passive immunity has worn off, such an animal possesses no active immunity against subsequent infection. We have reinfected rabbits of this type at from 15 to 60 days following original infection and in no case have we observed more than a "one-plus" immunity. In several cases the immunity was entirely negative.

If treatment is delayed until the 48th or 50th hour it does not appear to interfere with the development of active immunity, for such cases show the characteristic "three-plus" immunity after recovery.

This same principle has been demonstrated in animals given a single vaccination of washed heat-killed pneumococci. No active immunity develops in such cases if they are given, within 24 hours after vaccination, an amount of heterologous immune serum corresponding to an effective dose for that hour.

It has been suggested that this arrest of developing immunity may be concerned with the lack of immunizing power of the substances resulting from the proteolytic digestion of the phagocyted bacteria. It seems certain that the phagocytic activity of circulating leucocytes is increased by the agency of the immune serum and in the case of early treatment it might be reasoned that the bacteria or their antigenic fractions do not reach the tissues in form suitable for stimulating antibody production.

If cases are treated with heterologous immune serum in amounts insufficient to produce therapeutic effect (subeffective) a certain

number of animals recover spontaneously after a typical and severe course. In these instances the introduction of subeffective amounts of serum had not interfered with the development of immunity and these cases show the same immune character as do the untreated cases.

If an homologous antipneumococcic serum is used for arresting the course of the disease, and the rabbits are subsequently tested for immunity, the results differ from those obtained when heterologous serum is used. These animals, treated with the homologous serum, show a definite (+++) immunity following recovery. This immunity persists certainly for over a month at a high level and in one case a slight immunity (+) remained at 9 months. At this time there is no method by which it can be determined whether this result is due to an active immunity or to a prolonged and effective passive immunity. In this connection it is of interest to recall that Park (5) has reported a similar finding as regards diphtheria antitoxin, for he found that a guinea pig receiving 10 units of diphtheria antitoxin prepared in an immunized guinea pig remained immune for from 6 to 8 months, while a guinea pig receiving 10 units of antitoxin made in an immunized horse, remained immune for only 2 or 3 weeks.

Active Immunity and Agglutinins.—In twenty-eight cases of complete immunity to reinfection we have observed circulating agglutinins in about half. Partially immune animals have also been observed to show circulating agglutinins, even in as high a titer as 1–40. It is not easy to appraise the significance of these results, which however indicate quite clearly that the absence of circulating agglutinins does not signify the lack of immunity, and conversely it is also probable that the presence of circulating agglutinins does not indicate that the animal is completely resistant to infection.

Active Immunity and the Mouse-Protective Value of the Serum.—In a series of some twenty-five recovered rabbits we have correlated the immunity against infection with the protective value for mice of serum taken at that time. Animals whose sera are protective have always shown some degree of immunity to infection and in most instances this immunity is complete. Animals which are entirely non-resistant to infection show no protective substance but it is also true that some rabbits which are completely immune may possess serum with no protective value.

From the protective property alone, no conclusion is justified as to the immunity possessed by the animal. There remains only one method of testing the immunity of the experimental animal and that is to reinfect it.

Active Immunity after Vaccination with Killed Pneumococci.—It has been stated above that the "hyperimmunized" rabbits usually show a complete immunity (++++) against infection if such a test is carried out during the time that the antibody titer is high.

In our preceding paper we had occasion to use single vaccination as a means of studying the possible significance of the crisis. The knowledge that a single vaccination gave rise within 5 days to an immunity sufficient to protect an animal against infection has continued to interest us in its possible application to the disease in man. For this reason we have begun to study the immunity acquired in rabbits as a result of a single vaccination with heat-killed pneumococci. For routine intravenous vaccination we have used 10 cc. of a suspension, of washed heat-killed pneumococci, the density of which was 1.2 cm. (as measured by the method of Gates (6)). This is an extremely heavy suspension.

If the vaccinated rabbits are tested at 5 days by the usual method of intradermal inoculation of 0.2 cc. of undiluted broth culture, a three- or four-plus immunity is observed, in that respect being quite analogous to the convalescent case. In another sense the one-vaccination animal is unlike the convalescent for its acquired immunity wears off somewhat more quickly. In only one instance, of eight cases, did it persist in maximum degree for as long as 2 months.

The development of protective substances (for mice) in the rabbit has been quite thoroughly studied by Armstrong (7). In many of his experiments he observed a rise of protective substances within 3 to 5 days after a single injection of pneumococcus vaccine. We have confirmed that result. In Armstrong's series there was a gradual drop of circulating protective substances in from 8 days to several weeks; these intervals he held to be somewhat proportional to the amount of vaccine used. In our experiments this drop of protective substances has been irregular and apparently independent of the dosage.

The development of protective substances and immunity during a

period of 5 or 6 days following vaccination seems to be a characteristic of the pneumococcus rather than of the species of the animal used. We have found that mice develop an active immunity against infection within 4 or 5 days after a single injection of vaccine and this is possibly the fundamental reason that makes it unnecessary to observe mice in protection experiments for longer than that period of time. Through the kindness of Drs. White and Robinson of the Massachusetts Antitoxin and Vaccine Laboratory it has been possible to study the development of protective substances in horses after a single injection of heat-killed pneumococci. The sera of these horses were not examined on the 5th day but on the 6th protective substances were found in each of two cases. The crisis in human lobar pneumonia at 5 to 7 days must also be correlated with this characteristic period of time.

The Possible Application of These Findings.—In the foregoing paragraphs it has been established that, in rabbits, active immunity, whether acquired in disease or artificially by vaccination, is not of exceedingly long duration, and even at its height is unable in all cases effectively to overcome overwhelming doses of organisms immediately (the typical three-plus immunity). These facts are comparable to the present theories regarding active immunity in human lobar pneumonia.

There have been attempts, especially by Lister (8), and by Cecil and Austin (9), to use vaccination as a means of prophylaxis. Considering the difficulties in the preparation of a polyvalent vaccine and the apparently low antigenic properties of Type III, the results reported by these workers were very encouraging.

Although our experiments have been entirely with Type I and with experimental animals, it would seem that if vaccination could be applied in a systematic way to a susceptible population throughout the "pneumonia season" it should yield results. In such an application the two factors that must be especially considered are (*a*) the polyvalent character of the vaccine and (*b*) the shortness of the immune phase conferred by vaccination. It would seem possible to rule out the second point by the use of single subcutaneous injections of vaccine at 1- or 2-month intervals during the season of highest pneumonia morbidity.

SUMMARY.

1. The continuation of our experiments with intradermal Type I pneumococcus infection in rabbits has furnished further evidence of the marked analogies between this condition and that of human lobar pneumonia.

2. It has been found that the amount of antiserum necessary for successful therapy increases as the disease progresses, and that this progression has a definite mathematical character. Such a condition, it seems, can only be caused by a progressive accumulation of some toxic or antagonistic substance, the exact nature of which is not known.

3. Various lots of antipneumococcus sera have been tested for their therapeutic properties. The results from seven such sera show that this therapeutic value does not parallel the mouse-protective value. It is suggested that the rabbit technic may prove useful for the routine comparison and standardization of antipneumococcus sera since it represents a simple method for determining that property for which the serum is to be utilized.

4. The effect of non-specific therapy in this condition has been determined to be a transient disappearance from the blood stream of circulating organisms. This result was obtained with such heterologous materials as normal horse serum and typhoid vaccine but not with the homologous normal rabbit serum.

5. Rabbits recovering from the intradermal disease without treatment or with such inadequate treatment that the disease runs its normal course, were shown to have a definite though not permanent immunity. Cases in which the disease had been arrested at 24 hours by effective therapy with heterologous immune serum showed no immunity after the early disappearance of the passively administered elements. Cases which were brought to early recovery with immune homologous serum did show a definite immunity comparable to that which was developed in other animals as the result of an untreated course of the disease.

6. The immunity conferred by single and multiple vaccination is reported. The possibility of the application of such methods in the pneumonias of man is discussed and a method for such an application is suggested.

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THE RÔLE OF STREPTOCOCCI IN EXPERIMENTAL POLIOMYELITIS OF THE MONKEY.

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Within the last 12 years, several investigators have reported the isolation of streptococci from poliomyelitic tissues of man and of animals. These workers, in view of the ease with which the streptococci could be recovered and the supposed fulfillment by the organisms of one or all of Koch's postulates, believe that the streptococci are either the incitants of, or are etiologically related to poliomyelitis.[1–4] It was stated, furthermore, that the microorganisms after repeated injection into horses, can produce a serum which has neutralizing, protective, and therapeutic properties in poliomyelitis.[5] Other experimenters, however, have failed to confirm this opinion.

Thus Bull[6] reported that streptococci found in cultures of brain and spinal cord from poliomyelitic monkeys induced in monkeys neither the clinical signs

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nor the histopathological lesions of the experimental disease which follows inoculation of the filtered poliomyelitic virus. Nor were monkeys protected against filtered virus after recovery from the effects of injection of streptococci. Bull also cultivated streptococci from the tonsils of man—a tissue frequently employed by others[1,2,4] as a source of the supposed cocci of poliomyelitis. Streptococci from the tonsils of 32 patients with poliomyelitis and similar bacteria from the tonsils of non-poliomyelitic patients showed no differences in their respective action in laboratory animals. Bull, therefore, regarded the streptococci as secondary bacterial invaders of nervous tissue.

Smillie[7] concluded that the streptococcus could not be implicated in the pathology of the poliomyelitic process. He found that it was a common contaminant or a secondary invader: streptococci occurred in animals which were etherized while moribund, or which had died some hours before autopsy, but were absent in virus-infected animals which were killed while still relatively strong. Amoss[8] maintained a similar attitude.

With reference to the specific neutralization of the poliomyelitic virus by streptococcus immune serum, reported by Rosenow,[5] by Nuzum and coworkers,[5] Amoss and Eberson[9] found that such serum had neither neutralizing powers *in vitro* nor *in vivo*, or any therapeutic property in monkeys.

Finally, Flexner and his coworkers,[10] Levaditi and Landsteiner,[11] and others[12] have found that the effects of the filtrable poliomyelitic virus could not be ascribed to the action of ordinary bacteria.

The discrepancy in the results of different investigators and the recent increased interest in the use of antistreptococcic serum for the prevention and treatment of infantile paralysis, led us to a restudy of cultivation of poliomyelitic tissues. The study concerned especially the source of streptococci and their relation to the etiology of the disease. In general, it included a comparison of strains of streptococci isolated from monkeys affected with experimental poliomyelitis;

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with 3 strains of microorganisms generously supplied by Dr. Rosenow, and derived from poliomyelitic tissues; with 1 strain obtained from the American Type Culture Collection, also derived from poliomyelitis; and with 3 cultures of Rosenow poliomyelitic streptococci supplied by Eli Lilly and Company, whose kindness is gratefully acknowledged. In addition, cultures were made from the central nervous systems of poliomyelitic monkeys, with certain media recommended by Rosenow and others, media in which they obtained the so called poliomyelitic streptococcus. In view of the findings of two of the writers (Long and Olitsky[13]) that in so far as herpes virus encephalitis of rabbits and guinea pigs is concerned, the process of grinding permits contamination of tissues with streptococci and other ordinary bacteria, we cultured both fragments and emulsions of ground material from the same brain. Furthermore, cultivation tests were made with portions of the same brain in different rooms so as to check the results of one series against those of the other.

Mode of Procedure.

The materials cultured, the media and methods employed were as follows:

Source of Virus.—The poliomyelitic virus used consisted of the brains of 17 monkeys which showed the typical signs and pathological lesions of experimental poliomyelitis induced by the injection of either the M.A. strain[14] of poliomyelitic virus, or virus supplied us by Dr. Aycock.[15] The animals were killed at a time when the characteristic flaccid paralyses were clearly evident,[16] and tissues were removed for culture. In a few instances, the tissues were procured after the animal died from the typical experimental disease. In all cases, the diagnosis was checked by finding characteristic histopathological changes in the brain and spinal cord.[17] In addition the brain of a monkey which died from a non-poliomyelitic affection was also cultured.

The brains were removed from the monkeys under sterile precautions, and when fragments were used for cultures, 5 mm. cubes of tissue were cut for the purpose. When emulsions were employed, a portion of the brain was ground by

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[13] Olitsky, P. K., and Long, P. H., *J. Exp. Med.*, 1928, xlviii, 199.

[14] Flexner, S., Clark, P. F., and Amoss, H. L., *J. Exp. Med.*, 1914, xix, 195.

[15] This strain was more active in monkeys than the M.A. strain.

[16] Full ether anesthesia was used in all experiments.

[17] Flexner, S., and Lewis, P. A., *J. Exp. Med.*, 1910, xii, 227.

means of a sterile mortar and pestle. To this, physiological saline solution was added to make a 10 per cent suspension. About 1 cc. of the suspension comprised the inoculum. It is to be emphasized that all procedures were carried out under the strict conditions underlying sterile bacteriological technique.

Media.—The media employed consisted of:

(1) 5 per cent rabbit's blood, beef infusion 1 per cent dextrose or plain agar in Petri dishes.

(2) Chopped meat medium, prepared according to the directions given by Evans.[18] The medium was used because it was employed by the latter to recover streptococci from epidemic encephalitis in man and experimental encephalitis in monkeys and rabbits.

(3) Ascitic fluid-dextrose broth medium in long tubes. The medium was prepared according to the directions of Rosenow and Towne.[19]

(4) Smith-Noguchi medium based on the principles outlined by Gates and Olitsky.[20]

(5) Hartley's modification of Douglas' tryptic digest broth.[21] The special broth served as a basis for the study of toxins of the streptococci isolated during the course of the experiments.

(6) Beef infusion broth either plain or containing 1 per cent dextrose, for the preparation of agglutinogens and for a study of cultural reactions.

(7) Sterile peptone solution containing the Andrade indicator and respectively 1 per cent of dextrose, maltose, lactose, saccharose, raffinose, salicin, inulin, and mannitol for fermentation reactions.

Plan of Experiments and Technique.—For the purpose of ascertaining the origin of microorganisms in the cultures, that is, whether they were derived from the tissues themselves or from sources outside the tissues, experiments were designed as follows: (*a*) the same material was cultured in different media all of which were favorable for the growth of streptococci; (*b*) one brain was subdivided under sterile precautions into two parts of which one portion was used for cultures of fragments and the other for cultures of emulsions; (*c*) to eliminate the personal factor in the results obtained, two of the writers separately cultured portions of the same material; (*d*) one series of tests was made under the usual conditions of sterility, that is, under a hood in the room used for routine work. Another series was made under extraordinary conditions of sterility: cultures were performed in a manner similar to that employed by Dr. Carrel at The Rockefeller Institute for inoculation and transplantation of living tissue cells. The worker clothed himself in sterilized gown, hood, face mask, and rubber gloves. The media,

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[18] Evans, A. C., *Pub. Health Rep., U. S. P. H.*, 1927, xlii, 171.

[19] Rosenow, E. C., and Towne, E. B., *J. Med. Research*, 1917, xxxvi (N.S. xxxi), 175.

[20] Gates, F. L., and Olitsky, P. K., *J. Exp. Med.*, 1921, xxxiii, 51.

[21] Hartley, P., *J. Path. and Bact.*, 1922, xxv, 479.

material for cultivation, and instruments for cutting and grinding tissues were kept in sterile towels and treated as is done in modern surgical aseptic procedures. The room was especially adapted for elimination of air contaminants. It had no connection with the outside air and prior to the entry of the worker, motor-driven, powerful water sprays were put into action for the purpose of clearing the atmosphere of particles. The effectiveness of the procedures is exemplified by the fact that on one occasion blood agar medium in Petri dishes kept open for 35 minutes revealed no colonies of microorganisms.

All cultures were incubated at 37°C. and whenever any tube showed evidence of growth, it was examined; negative tubes were retained for 14 days before a final reading was made. It may be stated here that growth of the bacteria occurred as a rule from 18 to 72 hours after inoculation.

Cultivation Experiments.

Emulsions.—The first series of experiments comprised cultures of emulsions of brains from 18 monkeys in chopped meat medium and in ascitic dextrose broth. These were made by two of the writers, one working under the hood in the room usually employed for routine work, and the other in the special room already described, one worker alternating with the other in different tests. In two of the experiments a third person substituted for one of the original group.

The results of this experiment indicate that when the cerebral tissue was ground and emulsified and this material used for culture, a number of microorganisms, of different species, could be recovered. The number but not the species could be influenced by environmental conditions: when the usual, although strict sterile precautions were followed, the percentage of tubes of ascitic dextrose broth medium positive was 60, and of chopped meat medium, 57. When extraordinary care for sterility was employed, the positive tubes were respectively 21 and 13 per cent of all in each series.

Further analyses of Tables I and II show that the bacteria commonly encountered in the cultures of poliomyelitic brain emulsions in ascitic broth and meat media were diphtheroids, staphylococci, non-hemolytic streptococci, and spore-bearing rods. The same species were found on the medium in the Petri dishes exposed to the atmospheres of the two environments in which the cultures were made. The average time of exposure was about 30 minutes, that is, during the course of the setting up of a particular series of cultures. The average number of colonies on a plate exposed under the hood was

TABLE I.

Emulsions of Brain Cultured in Ascitic Dextrose Broth.

Monkey No.	Status	No. tubes		No. negative		No. positive		Non-hemolytic streptococci		Diphtheroids		Staphylococci		Spore-bearing rods		Miscellaneous		No. of colonies on opened plates	
		H	C	H	C	H	C	H	C	H	C	H	C	H	C	H	C	H	C
1	Experimental poliomyelitis	6		0		6		4		0		3 (*a*)		0		0			
2	" "	9		5		4				2		2		0		0			
3	" "	6	6	1	6	5	0	0	0	4	0	0	0	0	0	1 (*b*)	0		I = 0
4	" "	6	6	4	3	2	3	0	0	1	0	1	3	0	0	0	0	I(*c*) = 25	I = 6
																		II = 17	II = 10
5	" "	5	6	0	6	5	0	0	0	4	0	1	0	0	0	0	0	I = 59	I = 2
																		II = 47	II = 2
6	" "	5	6	3	5	2	1	0	0	2	1	0	0	0	0	0	0	I = 52	I = 2
																		II = 42	II = 4
7	" "	6	5	4	4	2	1	1	0	0	1	1	0	0	0	0	0	I = 16	I = 2
																		II = 16	II = 6
8	Normal	6		2		4		1		1		0		1		1 (*d*)		I = 18	
																		II = 16	
9	Experimental poliomyelitis	5	6	3	6	2	0	0	0	0	0	2	0	0	0	0	0	I = 14	I = 5
																		II = 8	II = 0
10	" "	6	6	4	0	2	6	0	6	1	0	1	0	0	0	0	0	I = 16	I = 4
																		II = 11	II = 2
11	" "	6		2		4		0		3		1		0		0		I = not counted	
																		II = 12	

12	"	"	6	6	1	6	5	0	0	0	5	0	1	0	0	0	0	0	I = 12 II = 16	I = 1 II = 12
13	"	"	6	6	0	5	6	1	0	0	5	0	0	1	0	0	1 (*e*)	0	I = 35 II = 37	I = 0 II = 0
14	"	"	6	6	2	4	4	2	0	0	2	2	1	0	1	0	0	0	I = 17 II = 15	I = 3 II = 0
15	"	"	6		5		1		0		0		0		0		1 (*f*)		I = 12	
16	"	"	6	6	5	5	1	1	0	0	0	1	1	0	0	0	0	0	I = 8 II = 10	I = 0 II = 1
17	"	"	6	6	0	6	6	0	0	0	3	0	3	0	0	0	0	0	I = 12 II = 12	
18	"	"	6		2		4		1		3 (*g*)		0		0		1 (*h*)		I = 14 II = 7	
Total 18			108	71	43	56	65	15	7	6	36	5	18	4	2	0	5	0		
	Per cent =				39+	78+	60+	21+					Average No. of colonies =						20+	3−

(*a*) In one case admixed with streptococci. (*b*) *M. tetragenus*. (*c*) No. of plate indicated by Roman numeral. The variations in numbers of colonies is due to length of time of exposure to the air which lasted throughout the period of the test. (*d*) Diplobacillus. (*e*) Streptobacillus. (*f*) Hemolytic streptococcus. (*g*) In one case admixed with streptococci. (*h*) Mould. H = cultures made in room used for routine work; C = cultures made in special (Dr. Carrel's) room.

about 20 and in Dr. Carrel's tissue culture room, about 3. There was evidently a correlation between the number of positive cultures with the number of bacteria in the air.

TABLE II.

Emulsions of Brain Cultured in Chopped Meat Medium.

Monkey No. (a)	No. tubes		No. negative tubes		No. positive tubes		Non-hemolytic streptococci		Diphtheroids		Staphylococci		Spore-bearing rods		Miscellaneous	
	H	C	H	C	H	C	H	C	H	C	H	C	H	C	H	C
1	6		0		6		4		1		1		0		0	
2	9		3		6		1		2 (*b*)		4 (*c*)		1 (*b*)		0	
3	6	6	1	6	5	0	1	0	3	0	0	0	0	0	1 (*d*)	0
4	6	6	5	4	1	2	0	0	0	0	1	2	0	0	0	0
5	5	7	1	7	4	0	0	0	4	0	0	0	0	0	0	0
6	5	6	0	6	5	0	0	0	1	0	3	0	1	0	0	0
7	6	5	2	4	4	1	1	0	1	1	1	0	1	0	0	0
9	5	6	4	5	1	1	0	0	0	0	1	0	0	1	0	0
10	6	6	4	2	2	4	0	4	0	0	2	0	0	0	0	0
11	6		5		1		0		1		0		0		0	
12	6	6	1	6	5	0	0	0	0	0	5	0	0	0	0	0
13	6	6	4	6	2	0	0	0	2	0	0	0	0	0	0	0
14	6	6	6	6	0	0	0	0	0	0	0	0	0	0	0	0
15	6		1		5		0		1		3		0		1 (*e*)	
16	6	6	4	5	2	1	0	0	2	1	0	0	0	0	0	0
17	6		0		6		0		2		4		0		0	
Total 16	96	66	41	57	55	9	7	4	20	2	25	2	3	1	2	0
Per cent =			42+	86+	57+	13+										

(*a*) For status of monkeys see Table I. Since these tests were made at the same time as those recorded in Table I, the control air cultures are not repeated here. (*b*) In one instance admixed with spore-bearing rod. (*c*) In one instance admixed with streptococci. (*d*) *M. tetragenus*. (*e*) Streptothrix. H and C as in Table I.

In addition it will be noted that although two portions of the same poliomyelitic or non-poliomyelitic monkey brain were employed, the results of cultures of each portion were in most instances wholly dissimilar. A striking example is that of Monkey 10. In one environment, 10 of 12 tubes of medium yielded non-hemolytic streptococci

(C, Tables I and II); in another room, of 12 tubes, 1 tube showed diphtheroids, 3 tubes staphylococci, and none revealed streptococci (H, Tables I and II).

Since the brains for culture were removed from monkeys while most of them were still relatively strong, although in different stages of flaccid paralyses, the notion that the bacteria were agonal invaders was not tenable. Moreover, the ground brain of the non-poliomyelitic monkey which was cultured at autopsy in ascitic broth also exhibited in different tubes diphtheroids, spore-bearing rods, diplobacilli, and non-hemolytic streptococci.

TABLE III.

Cultures of Fragments of Monkey Brain in Ascitic Broth and Chopped Meat Medium.

Monkey No.	Status	No. tubes chopped meat medium		No. tubes ascitic dextrose broth		No. of tubes positive in the meat medium		No. of tubes positive in the broth	
		H	C	H	C	H	C	H	C
2	Experimental poliomyelitis	9				0			
7	" "	6	5	5	6	0	0	0	0
8	Normal	6		6		0		0	
9	Experimental poliomyelitis	5	6	5	6	0	0	0	0
16	" "	6			6	0			0
17	" "	6			6	0			0
18	" "			6				1 (*a*)	
Total 7		38	11	22	24	0	0	1	0

Total No. tubes 95 Total positive 1 tube

(*a*) Diphtheroids. H and C as in Table I.

In general, the results of cultivation of emulsions of ground poliomyelitic and non-poliomyelitic cerebral tissues of monkeys parallel closely those of cultivation from herpes virus encephalitis of rabbits and guinea pigs, already reported.[13] In the last mentioned disease, it was shown that the various bacteria of common species, including the streptococci, were contaminants introduced during the process of grinding. Furthermore, in herpes virus encephalitis no growth of microorganisms was obtained when fragments, instead of ground

material, were used. How this applies in turn to poliomyelitic brain cultures is illustrated by Table III.

Fragments.—7 of the monkey brains which were cultured in the form of ground material were also cultured in the form of fragments.

Table III shows that of a total number of 95 tubes containing ascitic dextrose broth or chopped meat medium and fragments of monkey brains, 6 poliomyelitic and 1 non-poliomyelitic, only 1 exhibited growth. The positive tube contained diphtheroids. If a comparison be made with the cultures of emulsions of the same brains and the same media (Tables I and II), it will be plain that with poliomyelitic brains just as with herpes virus encephalitis brains, cultures of ordinary bacteria occurred as a consequence of emulsifying the cerebral tissues. Again it should be emphasized that the results shown in all three tables were derived from portions of the same brain.

In an additional experiment, a poliomyelitic brain from Monkey 15 was cut into fragments, the fragments placed in broth at 37°C. for 24 hours, and then transferred to 6 tubes of ascitic dextrose broth and 6 tubes of chopped meat medium. The latter medium was, after 14 days incubation, negative but the ascitic broth cultures showed nonhemolytic streptococci in 2 tubes, staphylococcus, diphtheroids, and streptothrix in single respective tubes. This test was made in the routine culture room. It should be compared with the findings in the same material as given in Tables I and II. It is obvious that the results in this case depended on the additional handling of the brain.

To summarize, a variety of ordinary bacteria including nonhemolytic streptococci can be recovered from cultures of poliomyelitic monkey brains. They appear irregularly in tests with different portions of the same brain, occurring sometimes in one favorable medium but not in another, frequently in one room but not in a second, often when only the usual bacteriological technique for sterility is followed but much less often when extraordinary precautions for sterility are maintained, and generally in emulsions of the brain but not in its fragments. Furthermore, the bacteria are similar to those often recovered from the air of the place in which the cultures are made. What has been said, therefore, of herpes virus encephalitis can be stated again as applying to experimental poliomyelitis in monkeys, namely, the bacteria are introduced into the material for culture during grinding.[13]

Smith-Noguchi Medium.—This opinion is further supported by results of cultures in the Smith-Noguchi medium, although the primary purpose of this series of cultivation tests was the recovery of the globoid bodies of Flexner and Noguchi.[22] The cultures were made with fragments of monkey brain in ordinary Smith-Noguchi medium[20]

TABLE IV.

Cultivation of Fragments in Smith-Noguchi Medium.

Monkey No. (*a*)	No. of tubes		No. of tubes positive		Diphtheroids		Non-hemolytic streptococci		Staphylococci		Miscellaneous		Globoid bodies	
	O	B	O	B	O	B	O	B	O	B	O	B	O	B
3	5	5	1	0	1	0	0	0	0	0	0	0	0	0
4	6	6	1	6	0	2	0	0	1	0	0	1 (*b*)	0	3
5	5	5	4	0	1	0	1	0	1	0	1 (*c*)	0	0	0
6	5	5	2	1	1	0	0	0	1	0	0	1 (*d*)	0	0
7	6	6	1	2	1	1	0	0	0	0	0	1 (*c*)	0	0
8	6	6	4	1	2	0	0	1	0	0	2 (*c*)	0	0	0
9	6	6	0	0	0	0	0	0	0	0	0	0	0	0
10	6	6	0	0	0	0	0	0	0	0	0	0	0	0
11	6	6	0	1	0	1	0	0	0	0	0	0	0	0
12	6	6	0	0	0	0	0	0	0	0	0	0	0	0
13	4	5	1	1	0	0	0	0	0	0	0	0	1	1
14	6	6	0	0	0	0	0	0	0	0	0	0	0	0
16	6	6	0	0	0	0	0	0	0	0	0	0	0	0
17	6	6	0	1	0	1	0	0	0	0	0	0	0	0
Total 14	79	80	14	13	6	5	1	1	3	0	3	3	1	4

(*a*) The status of these monkeys is given in Table I. Compare with Tables I to III for results on aerobic cultivation. (*b*) Streptothrix. (*c*) Spore-bearing rod. (*d*) Diplococcus. O = Ordinary Smith-Noguchi medium and B = Boëz' apparatus.

and similar medium having no petrolatum seal but placed in an anaerobic apparatus described by Boëz.[23] The results are given in Table IV.

There is general agreement on the difficulty of avoiding contamination in the Smith-Noguchi technique. In the series shown in Table IV 27 of 159 tubes exhibited different bacteria. The number of positives

[22] Flexner, S., and Noguchi, H., *J. Exp. Med.*, 1913, xviii, 461.

[23] Boëz, L., *J. Bact.*, 1927, xiii, 227.

might have differed if one instead of three workers had made all the tests. However, in the case of 2 monkeys (Nos. 5 and 6), emulsions of ground brain were also planted in Smith-Noguchi medium; 10 of 12 tubes showed different bacteria.

With respect to the isolation of globoid bodies, it is to be noted that they were obtained from the poliomyelitis brains of 2 of the 14 monkeys (Table IV). In 1 case, 3 of 12 tubes were positive, and in the other 2 of 9 tubes. 1 culture in its first generation was inoculated intracerebrally into a monkey; no signs of experimental poliomyelitis developed.[24]

The globoid bodies were found to be distinct microorganisms but differed from the streptococci recovered in the tests and from those obtained from Rosenow. They were definitely anaerobic; indeed, in one instance, a transplant from a culture in ascitic agar kept in the dark at room temperature for about 9 years was still viable and anaerobic. On the other hand, the inoculation of streptococci into Smith-Noguchi medium failed to bring about their conversion to globoid type. 9 strains of streptococci, including 3 of Rosenow's series, planted in the medium and examined after 14 days incubation showed death of 2, somewhat smaller forms in 3, and no change in the remaining strains. Transfer of the survivors to aerobic medium yielded profuse growths of typical large cocci or coccoid forms. These conditions prevailed through 3 successive subplants in Smith-Noguchi medium at 14 day intervals.

Properties of the Streptococci.

31 strains of non-hemolytic streptococci were collected for comparative study. In this collection were 7 strains of Rosenow, all of which were stated to be derived from poliomyelitis;[25] 1 was recovered from "ground" broth and 3 from ground chopped meat

[24] In this connection it is of interest to record a repeated experiment in which fragments of poliomyelitic brain were placed in broth and kept for 14 days under aerobic and anaerobic conditions. At the end of this time, the fragments were removed and ground in saline solution and injected intracerebrally in monkeys. Neither the aerobic nor the anaerobic material was found to be active.

[25] 3 of the strains were given us by Dr. Rosenow, 3 by Eli Lilly and Company, and 1 was obtained from the American Type Culture Collection.

medium, already described;[13] 2 from herpes virus encephalitis in rabbits;[13] 2 from normal guinea pig brain;[13] 5 from the air in the rooms in which the cultures were made; 1 from a non-poliomyelitic monkey brain; and 10 from poliomyelitic monkey brains. In addition there were included 1 strain of enterococcus and 1 of hemolytic streptococcus—the latter being recovered from the air.

Cultural Characteristics.—With few exceptions, the non-hemolytic streptococci produced on rabbit blood agar small, round, greenish tinged colonies with a very narrow zone of clearing; exceptional colonies did not produce this zone. On rabbit blood dextrose agar the colonies precipitated the medium to dark brown, just underneath and surrounding the colony. In fluid medium, consisting of chopped meat broth, ascitic dextrose broth, dextrose and plain broth, all the streptococci grew profusely within 18 to 48 hours. The dextrose media showed a granular appearance along the sides and a heavy, whitish precipitate; the other media revealed either granular or diffuse, but also luxuriant growths.

Morphologically long chained forms predominated but 2 of Rosenow's cultures and some selected streptococci obtained from the air and from other non-poliomyelitic sources revealed diplococcus forms often in short chains. All showed pleomorphism, and when a long chained form was inoculated into a rabbit, the tissues would usually reveal diplococcus forms; this happened irrespective of the source of the culture.

Tests for fermentation were made on 24 cultures of the non-hemolytic streptococci. Four distinct groups containing cultures having identical reactions could be determined; the reactions of each group as a whole, however, differed from those of the others. A fifth group contained 11 heterogeneous strains which were unrelated to any of the others. All cultures were classified as follows:

Group I.	1 strain of streptococcus obtained from air (Dr. Carrel's room).
	2 strains of Rosenow's series given us by Eli Lilly and Company.
Group II.	1 strain of streptococcus obtained from air (hood).
	1 strain from non-poliomyelitic brain.
	1 strain from poliomyelitic brain.
Group III.	1 strain (No. 349) of Rosenow's series from American Type Culture Collection.
	1 strain obtained from Rosenow.

Group IV. 3 strains from poliomyelitic brains.
2 strains obtained from Rosenow.

Group V. Heterogeneous. Includes 2 strains obtained from the air, 1 from Eli Lilly and Company, and 8 from poliomyelitic monkey brains. In this group only one culture fermented mannitol; 3, salicin; 6, inulin (3 faintly so); 3, raffinose; and all fermented dextrose, maltose, lactose, and saccharose.

It is noteworthy that there was no uniformity in fermentation reactions. Furthermore, when 2 or more cultures were recovered from the same brain, the individual growths showed different responses to the tests.

Serological Reactions.—With regard to direct and cross-agglutination reactions with rabbit immune serum, the different cultures of non-hemolytic streptococci, irrespective of source, also showed a marked heterogenicity.

Rabbits were immunized by the method of injecting intravenously first dead then living cultures. 3 to 4 inoculations were given at daily intervals and after a rest of 4 to 5 days, the daily series was continued, proceeding thus for over a month. 10 days after the last injection, the rabbits were exsanguinated. The blood serum then revealed agglutinins in dilutions of 1:320 to 1:5120 to homologous strains. In only one instance was the titre 1:40.

As antigens for the preparation of the rabbit serum, 10 strains were used, of which 7 were directly or indirectly obtained from Rosenow and were classified by him as "poliomyelitic" streptococci; 2 were recovered from cultures of "ground" broth or chopped meat medium;[13] and 1 was derived from cultures of poliomyelitic monkey brain. Agglutination tests were set up after the manner of Rosenow with the 10 sera and 25 different cultures. The latter included an enterococcus, a *Micrococcus tetragenus*, a hemolytic streptococcus, and 22 strains of non-hemolytic streptococci derived from the air, from media, from non-poliomyelitic guinea pig and monkey brains, and from poliomyelitic monkey brains.

A summary of the results reveals that 3 of the sera prepared with 3 cultures, 2 of which were derived respectively from "ground" media and 1 from a poliomyelitic monkey brain, agglutinated only the homologous cultures. The remaining 7 sera agglutinated, apart from the homologous strains, very few of the 25 cultures put to test. The latter 7 sera were all prepared with different strains ultimately derived from Rosenow. The positive results with these sera follow:

Serum 809, positive only with 2 strains of streptococci obtained from poliomyelitic monkey brains, in dilutions of 1:640 and 1:2560; and Serum 866, positive only with 1 similar strain (1:160). Serum 2254, positive only with 2 other Rosenow strains (1:160 and 1:320). Thus also Serum 349 (1:160 and

1:1280). Serum 3002, positive only with 1 other Rosenow strain (1:40); similar to this was Serum 3007 (1:1280). Serum 3005, positive only with 1 other Rosenow serum (1:320) and with a non-hemolytic streptococcus obtained from ground normal guinea pig brain.

One may therefore conclude that from the view-point of serological reactions the non-hemolytic streptococci, whether obtained from poliomyelitic or other material, form a class of bacteria of dissimilar antigenic function. Furthermore, pooled serum from monkeys recovered from experimental poliomyelitis (and similar serum from normal monkeys, employed as control) failed to agglutinate any of the non-hemolytic streptococci, including Rosenow's 7 strains. Finally, a similar heterogenicity among the poliomyelitic and among the non-poliomyelitic streptococci was revealed by precipitin tests.

Skin Reactions in Rabbits.—No skin reactions were noted in rabbits when the animals were injected intracutaneously with a filtrate from Hartley's modification of Douglas' tryptic digest broth[21] inoculated with Rosenow's strains of streptococci and incubated for 10 days, nor were skin reactions visible with lysates of these microorganisms prepared *in vivo* in immunized rabbits.

Pathogenicity for Rabbits.—It has been stated by Rosenow and others that rabbits dying of streptococcus infection showed signs and lesions identical with those of poliomyelitis in man and monkeys.[1,2,4]

36 rabbits were inoculated intracerebrally with 0.35 cc. to 0.4 cc. of 18 to 24 hour old broth cultures of non-hemolytic streptococci derived from the air, from non-poliomyelitic and poliomyelitic monkey brains. 18 different cultures were used and each was inoculated into 2 rabbits. The results are tabulated on page 446.

In spite of the fact that the filtrable virus of poliomyelitis cannot be successfully implanted on the nervous tissue of rabbits,[26] the streptococci, on the other hand, readily induced a reaction which is not characteristic of the virus of poliomyelitis but is typical of streptococcus infection. It is to be noted that the microorganisms derived from poliomyelitic and non-poliomyelitic tissues; or from the air; or, as has been previously reported,[13] from "ground" medium, and from ground normal or herpes virus-infected brain, show no distinctive effects in rabbits. Many of the cultures of streptococci,

[26] Flexner, S., and Lewis, P. A., *J. Am. Med. Assn.*, 1910, liv, 45.

obtained from different sources, are pathogenic for rabbits, producing purulent meningoencephalitis complicated by streptococcic septicemia, and few of these bacteria are apparently non-pathogenic. It is noteworthy that some rabbits are resistant to pathogenic strains —an observation also made in the studies on herpes virus encephalitis.[13]

Strain	Source	Results in each of 2 rabbits
3002 (*a*)	Rosenow (from poliomyelitic tissue)	1 died within 24 hrs.; the other survived
3005	" "	Both survived
3007	" "	" died within 24 hrs.
349	" "	1 died within 24 hrs.; the other survived
809	" "	Both died within 24 hrs.
866	" "	" " " " "
2254	" "	" survived
1	Experimental poliomyelitis of monkey	" died within 48 hrs.
3	" "	1 died within 48 hrs.; the other survived
6	" "	" "
7	" "	Both died within 24 hrs.
15	" "	" survived
10	" "	" died within 24 hrs.
10E5	" "	1 died on the 5th day; the other survived
8	Non-poliomyelitic monkey	Both died within 24 hrs.
Air C	Air Dr. Carrel's room	" " " " "
Air H	Air routine laboratory	" survived
Air H 2	" " "	1 died within 24 hrs.; the other survived

(*a*) Strains 3002, 3005, 3007 were obtained from Eli Lilly and Company; 349, from the American Type Culture Collection; and 809, 866, and 2254 directly from Dr. Rosenow.

The rabbits that died exhibited purulent meningoencephalitis. Their brains, and in most instances the heart's blood, yielded pure growths of streptococci. In respect to the action of streptococci in rabbits, we have confirmed in detail the previous findings of Bull,[6] to whose work the reader is referred for a comprehensive description of the signs and histopathology in injected animals. These experiments are also in complete agreement with those reported by Olitsky and Long on the rôle of streptococci in herpes virus encephalitis,[13] and the reader is also referred to this paper for a fuller account of the action of streptococci in rabbits.

DISCUSSION AND SUMMARY.

It is the opinion of Bull,[6] that the streptococci recovered from poliomyelitic tissues, while having no etiological or pathological relationship to the virus of poliomyelitis, occur as secondary invaders in the disease. Smillie[7] and Amoss[8] indicated that the bacteria may be agonal invaders.

The results of the experiments reported in this paper point to another source of the streptococci. They occur as contaminants which are introduced into the cultures during the process of grinding tissues. The source of the streptococcus may therefore be the air of the place in which the cultures are made. We have come to this conclusion because first, the tissues of which cultures yielded streptococci were derived from a number of monkeys with experimental poliomyelitis still in a vigorous state. Secondly, when the tissues were ground bacteria were noted much more frequently in their cultures than in those in which fragments of the same brains were used. Thirdly, microorganisms occurred more often in cultures made in the routine laboratory than in a special room where asepsis was carried to the extreme of a major surgical operation on man. Fourthly, streptococci were obtained from the air of the places where cultures were made. Finally, there is no correlation between the cultures of two portions of the same brain.

The streptococci occurred in some cultures in pure growth and in others admixed with other ordinary species of bacteria. The latter were often found, in turn, in pure culture and what applies to streptococci, as mentioned in the preceding paragraph, applies equally to the staphylococci, diphtheroids, spore-bearing rods, and other miscellaneous, familiar microorganisms.

We could not determine that there exists any etiological relation of the streptococci to poliomyelitis. The fermentation reactions of the microorganisms obtained from the air, from non-poliomyelitic and poliomyelitic monkey brains indicate that bacteria from any of these sources are markedly different. So also with the serological reactions of agglutination and precipitation. Furthermore no agglutination was observed when the serum of monkeys convalescent from experimental poliomyelitis was mixed with any of the streptococci

recovered or those received directly or indirectly from Rosenow. Moreover, the intracerebral injection with cultures, irrespective of their source, induced in rabbits a purulent type of meningoencephalitis, often associated with streptococcic septicemia. This result is at marked variance with any known effects of the true filtrable virus of poliomyelitis in man and in the monkey.

VIRUS NEUTRALIZATION EXPERIMENTS WITH ROSENOW'S AND PETTIT'S ANTIPOLIOMYELITIC SERA.

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During the past decade three types of antipoliomyelitic sera have been employed in the therapy of acute anterior poliomyelitis. These are, first, the sera of convalescent human poliomyelitis; second, sera from horses immunized against the streptococci supposed by Rosenow and others (1–12) to be etiologically related to poliomyelitis; third, the Pettit serum prepared at the Pasteur Institute (13). The last mentioned product consists of sheep serum or horse serum from animals supposedly immunized against poliomyelitis virus through repeated intravenous injections of emulsions of spinal cords of poliomyelitic monkeys. The experimental evidence upon which Rosenow and his coworkers base their claims for the efficacy of antistreptococcus poliomyelitis serum is discussed in another paper (14) and need not receive further mention at this moment. As regards the antipoliomyelitic serum of Pettit, it is sufficient to say that, so far as can be determined, the experimental evidence for its therapeutic value rests upon a single neutralization experiment of somewhat doubtful significance, as we shall later show. Since both the Rosenow and Pettit's serums have been more or less widely employed, it was deemed desirable to study this neutralizing action on the virus of poliomyelitis.

Amoss and Eberson (20) have already made a series of neutralizing tests with the Rosenow serum and failed to detect neutralizing properties. The Pettit serum was originally prepared from sheep "immunized" by intravenous injection with suspensions of poliomyelitic monkey cords. Later, "immune" horse serum was produced by a similar procedure (15). As previously mentioned, only one neutralization experiment is recorded. Two monkeys, A and B, received intracerebrally virus plus "immune" sheep serum and virus alone respectively. The monkey with the serum-virus mixture remained normal, but the monkey receiving virus

alone developed typical poliomyelitis. Since occasional neutralization of poliomyelitis virus by sheep sera is not at all unknown in this laboratory (21), we were inclined to suspect that the apparent favorable result obtained by Pettit rested upon some such chance neutralization; this was more probable since in the experiment reported the control neutralization set up was made with virus alone instead of with a mixture of virus plus "normal" sheep serum. This is an unsafe procedure in testing a heterologous serum. For this occasional virus neutralization by sera of non-susceptible animals, no immediate explanation is apparent.

TABLE I.

Series I.

Monkey	Intracerebral inoculum	Result*
1	Pettit antipoliomyelitis serum..... 0.9 cc. 5% Berkefeld filtrate poliomyelitis virus.... 0.3 cc.	Typical poliomyelitis
2	Rosenow antipoliomyelitis serum concentrated (Eli Lilly)..... 0.9 cc. 5% Berkefeld filtrate poliomyelitis virus.... 0.3 cc.	Typical poliomyelitis
3	Human convalescent serum *a*..... 0.9 cc. 5% Berkefeld filtrate poliomyelitis virus.... 0.3 cc.	Remained well
4	Human convalescent serum *b*..... 0.9 cc. 5% Berkefeld filtrate poliomyelitis virus.... 0.3 cc.	Remained well
5	Normal horse serum..... 0.9 cc. 5% Berkefeld filtrate poliomyelitis virus.... 0.3 cc.	Typical poliomyelitis
6	Normal human serum..... 0.9 cc. 5% Berkefeld filtrate poliomyelitis virus.... 0.3 cc.	Abortive poliomyelitis

* Confirmed by histological study.

Numerous clinical papers (15–19) are available, showing the supposed beneficial effect of the Pettit serum in the arrest of human poliomyelitis. In none is it possible for an unbiased reader to ascertain any certain evidence that the beneficial results attained were really due to the serum therapy. In fact, certain favorable results are claimed in late paralyzed cases well beyond the period of acute disease and even in cases of myelitis certainly clinically of non-poliomyelitic variety.

The experiments to be reported in the present paper are several. All were rigorously controlled.

Macacus rhesus monkeys were used as experimental animals. The poliomyelitis virus serving for infection was the Rockefeller Institute M.A. strain. The Rosenow serum was obtained from Eli Lilly and Company, the Pettit serum through the kindness of Dr. A. Pettit; a similar serum was later prepared by us in accordance with Pettit's original published method. As control sera we employed those of convalescent humans, convalescent monkeys, normal monkeys, normal horse, and normal sheep. The sera to be tested were mixed with either suspensions or Berkefeld filtrates of fresh virus, were incubated 2 hours, and allowed to stand overnight in the ice box. All animals were etherized and infected

TABLE II.

Series II.

Monkey	Intracerebral inoculum	Result
7	Pettit antipoliomyelitis serum..... 0.9 cc. 5% Berkefeld filtrate poliomyelitis virus.... 0.3 cc.	Remained well
8	Rosenow antipoliomyelitis serum unconcentrated (Eli Lilly)..... 0.9 cc. 5% Berkefeld filtrate poliomyelitis virus.... 0.3 cc.	Typical poliomyelitis
9	Human convalescent serum..... 0.9 cc. 5% Berkefeld filtrate poliomyelitis virus.... 0.3 cc.	Remained well
10	Monkey convalescent serum..... 0.9 cc. 5% Berkefeld filtrate poliomyelitis virus.... 0.3 cc.	Remained well
11	Normal horse serum..... 0.9 cc. 5% Berkefeld filtrate poliomyelitis virus.... 0.3 cc.	Typical poliomyelitis
12	Normal monkey serum..... 0.9 cc. 5% Berkefeld filtrate poliomyelitis virus.... 0.3 cc.	Typical poliomyelitis

intracerebrally. The results are best shown by Tables I to VI. We are concerned only with the development of definite paralytic poliomyelitis and not with the question of death or survival, degree of paralyses, or length of incubation periods. In small series these are deemed of doubtful significance.

It is evident from the three series of experiments summarized in Tables I to III that no virus neutralizations have occurred with Rosenow's serum—at least with the three different samples employed. The Pettit serum has neutralized, but the neutralization was inconstant. To test further this serum, the virus used was prepared as a

suspension rather than a Berkefeld filtrate. Pettit utilized suspensions in his own neutralization tests. Further results with both suspension and filtrate appear in Table IV. It is quite apparent that the Pettit serum does not consistently neutralize virus.

TABLE III.

Series III.

Monkey	Intracerebral inoculum	Result
13	Rosenow antipoliomyelitis serum..... 0.9 cc. 5% Berkefeld filtrate poliomyelitis virus.... 0.3 cc.	Typical poliomyelitis
14	Pettit antipoliomyelitis serum..... 0.9 cc. 5% Berkefeld filtrate poliomyelitis virus.... 0.3 cc.	Questionable poliomyelitis
15	Normal horse serum..... 0.9 cc. 5% Berkefeld filtrate poliomyelitis virus.... 0.3 cc.	Typical poliomyelitis

TABLE IV.

Series IV.

Monkey	Intracerebral inoculum	Result
16	Pettit antipoliomyelitis serum..... 0.9 cc. 5% suspension poliomyelitis virus..... 0.3 cc.	Typical poliomyelitis
17	Pettit antipoliomyelitis serum..... 0.9 cc. 5% Berkefeld filtrate poliomelitis virus..... 0.3 cc.	Questionable symptoms only. No paralysis
18	Pettit antipoliomyelitis serum..... 0.9 cc. 5% Berkefeld filtrate poliomyelitis virus.... 0.3 cc.	Typical poliomyelitis
19	Normal horse serum..... 0.9 cc. 5% Berkefeld filtrate poliomyelitis virus.... 0.3 cc.	Poliomyelitis (excitement, fatigue, prominent facial paralysis only)

Following these experiments, we determined to prepare sheep sera according to the method of Pettit. Three sheep were used. Previous to the first inoculation, the sera of these animals were tested for virus neutralization in monkeys. The results appear in Table V.

TABLE V.

Series V.

Monkey	Intracerebral inoculum	Result
20	Normal serum Sheep I..... 0.9 cc. 5% Berkefeld filtrate poliomyelitis virus 1965 and 1983..... 0.3 cc.	Remained well
21	Normal serum Sheep I..... 0.9 cc. 5% suspension poliomyelitis virus 1993..... 0.3 cc.	Typical poliomyelitis
22	Normal serum Sheep II..... 0.9 cc. 5% Berkefeld filtrate poliomyelitis virus 1965 and 1983..... 0.3 cc.	Typical poliomyelitis
23	Normal serum Sheep III..... 0.9 cc. 5% Berkefeld filtrate poliomyelitis virus 1993..... 0.3 cc.	Remained well
24	Normal monkey serum..... 0.9 cc. 5% suspension poliomyelitis virus..... 0.3 cc.	Typical poliomyelitis

TABLE VI.

Series VI.

Monkey	Intracerebral inoculum	Result
25	Normal serum Sheep II..... 0.9 cc. 5% suspension poliomyelitis virus..... 0.3 cc.	Remained well
26	"Immune" serum Sheep II..... 0.9 cc. 5% suspension poliomyelitis virus..... 0.3 cc.	Typical poliomyelitis
27	Normal serum Sheep III..... 0.9 cc. 5% suspension poliomyelitis virus..... 0.5 cc.	Remained well
28	"Immune" serum Sheep III.....0.9 cc. 5% suspension poliomyelitis virus .....0.5 cc.	Typical poliomyelitis
29	5% suspension poliomyelitis virus..... 0.3 cc.	Typical poliomyelitis

Thus it would appear that Sheep I serum, previous to any immunization, inhibited Berkefeld filtrate once and at another time failed to inhibit suspension; that Sheep II serum failed to inhibit

Berkefeld filtrate; that Sheep III serum inhibited Berkefeld filtrate. Sheep I was lost from intercurrent disease. Sheep II and III were given twenty-one biweekly, intravenous injections, each consisting of 5 cc. of 5 per cent virus suspension from fresh or glycerolated monkey spinal cords. The animals were bled after a brief rest, and the sera were again tested for neutralization or inhibition.

The lack of consistency in results is well shown by the fact that both normal sera inhibited or neutralized virus, whereas sera from the same animals after "immunization" failed to do so.

CONCLUSIONS.

1. The Rosenow antistreptococcic poliomyelitis serum concentrated or unconcentrated does not neutralize the virus of poliomyelitis as tested in monkeys.

2. The Pettit antipoliomyelitis horse serum neutralizes the virus only occasionally.

3. "Immune" sheep sera prepared according to the method of Pettit have not neutralized virus even when the normal sera of the same animal have given neutralization.

4. The reason for such chance neutralizations is obscure and should not be confused with the constant virus-neutralizing action of both human and monkey convalescent sera.

5. Experimental evidence affords no basis for the use of either the Rosenow or the Pettit serum in the therapy of poliomyelitis.

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